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The Relationship between Aortic Calcification and Osteoporosis in Postmenopausal Women

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The Relationship between Aortic Calcification and Osteoporosis in Postmenopausal Women

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I would like to dedicate this piece of work to my late Father,

Francis Ernest Edwards MBE.



Abstract

It is widely accepted that both osteoporosis (OP) and cardiovascular disease (CVD) are common conditions and are a major cause of morbidity and mortality within the ageing population. Traditionally they have been viewed as two separate conditions that increase with age and are therefore frequently seen in the same individual. In this large study of 462 postmenopausal women the primary objective is to investigate the relationship between OP and aortic calcification (AC); investigating associations between AC, BMD and regulators of bone remodelling that have been implicated in the pathogenesis of vascular calcification (VC) such as the Wnt signalling pathway. A secondary objective is to investigate the effects of bisphosphonates (BPs) on VC in postmenopausal women with low BMD, in a 2-year randomised controlled trial. This research is clinically important since no therapies are available that can reverse VC. Results will provide valuable information on whether BPs can be used to prevent or decrease AC in addition to improving BMD and reducing fracture risk. An important aspect of this study is to evaluate the novel application of simple, non-invasive imaging techniques for quantifying AC. Two methods including pulse wave velocity (PWV), an ultrasound method of assessing arterial stiffness, and lateral vertebral fracture assessment (VFA) scanning; an imaging method previously validated for the quantification of AC, will be compared to the gold-standard of computed tomography (CT).

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Glossary of Abbreviations

AA	Abdominal Aorta
AAC	Abdominal Aortic Calcification
AC	Aortic Calcification
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AMI	Acute Myocardial Infarction
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
AP	Anteroposterior
AU	Arbitrary Unit
ATP	Adenosine Triphosphate
AUC	Area Under Curve
AVC	Aortic Valve Calcification
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMI	Body Mass Index
BMP	Bone Morphogenetic Protein
BMU	Basic Multicellular Unit
BP	Bisphosphonate
CA	Carotid Artery
Ca/P	Calcium/Phosphate
CAD	Carotid Artery Disease
CHD	Coronary Heart Disease
CHF	Congestive Heart Failure
CI	Confidence Interval (%)
CIMT	Carotid Intima Media Thickness
CKD	Chronic Kidney Disease
CKD-MBD	Chronic Kidney Disease - Mineral Bone Disorder
COPD	Chronic Obstructive Pulmonary Disease
CRF	Clinical Risk Factors
CRP	C-Reactive Protein
CT	Computed Tomography
CTX	C-terminal Telopeptide
CV%	Coefficient of Variation

CVC	Cardiovascular Calcification
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
Dkk1	Dickkopf-related Protein 1
DM	Diabetes Mellitus
DVT	Deep Vein Thrombosis
DXA	Dual Energy X-ray Absorptiometry
EBCT	Electron Beam Computed Tomography
ECG	Electrocardiogram
eGFR	Estimated Glomerular Filtration Rate
ELISA	Enzyme-linked Immunosorbent Assay
EPC	Endothelial Progenitor Cells
ESR	Erythrocyte Sedimentation Rate
ESRD	End-Stage Renal Disease
FBC	Full Blood Count
FDA	Food and Drug Administration (USA)
FGF	Fibroblast Growth Factor
FoV	Field of View
FRAX	The Fracture Risk Assessment Tool
FSH	Follicle Stimulating Hormone
GI	Gastro Intestinal (Disease)
GIOP	Glucocorticoid Induced Osteoporosis
HDL	High Density Lipoprotein
HRT	Hormone Replacement Therapy
HU	Hounsfield Units
IA	Iliac Artery
IBD	Inflammatory Bowel Disease
IC	Intermittent Claudication
ICC	Intraclass Correlation Coefficient
IGF	Insulin-like Growth Factor
IHD	Ischaemic Heart Disease
IMT	Intima-Media Thickness
IQR	Interquartile Range
IRMER	Ionising Radiation (Medical Exposure) Regulations
IU	International Unit
IV	Intra Venous
LDL	Low Density Lipoprotein

LH	Luteinizing Hormone
LoA	Limit of Agreement
LRP-5/6	Low-Density Lipoprotein Receptor-Related Protein 5/6
LVH	Left Ventricular Hypertrophy
MAP	Mean Arterial Pressure
MDCT	Multi-detector Computed Tomography
MI	Myocardial Infarction
MRI	Magnetic Resonance Imaging
M-SCF	Macrophage Colony-stimulating Factor
NCBP	Nitrogen Containing Bisphosphonates
NHANES III	National Health and Nutrition Examination Survey
NHS	National Health Service (UK)
NMR	Nuclear Magnetic Resonance
Non NCBP	Non Nitrogen Containing Bisphosphonates
NPV	Negative Predictive Value
ON	Osteonectin
ONJ	Osteonecrosis of the Jaw
OP	Osteoporosis
OPG	Osteoprotegerin
OPN	Osteopontin
P1NP	Procollagen type 1 N-terminal Propeptide
PA	Posteroanterior
PAD	Peripheral Artery Disease
PAS	Physical Activity Status
PE	Pulmonary Embolism
PPV	Positive Predictive Value
pQCT	Peripheral Quantitative Computed Tomography
PR	Prevalence Ratio
PTH	Parathyroid Hormone
PWV	Pulse Wave Velocity
QCT	Quantitative Computed Tomography
QUS	Quantitative Ultrasound
RA	Rheumatoid Arthritis
RANK	Receptor Activator of Nuclear Factor kappa-B
RANKL	Receptor Activator of Nuclear Factor kappa-B Ligand
RCT	Randomised Controlled Trial
ROC	Receiver Operating Characteristics

ROI	Region of Interest
Runx2	Runt-related Transcription Factor 2
SBP	Systolic Blood Pressure
SCLE	Sclerostin
SD	Standard Deviation
SERM	Selective Estrogen receptor modulators
SFoV	Scan Field of View
SLE	Systemic Lupus Erythematosus
SPECT/CT	Single-photon Emission Computerised Tomography
TA	Thoracic Aorta
TB-BMC	Total Body Bone Mineral Content
TCS	Total Calcium Score
TIA	Transient Ischaemic Attack
TNF	Tumour Necrosis Factor
TSH	Thyroid Stimulating Hormone
UV	Ultra Violet
vBMD	Volumetric Bone Mineral Density
VC	Vascular Calcification
VEGF	Vascular Endothelial Growth Factor
VFA	Vertebral Fracture Assessment
VSMC	Vascular Smooth Muscle Cells
WHO	World Health Organisation
1 ^o HPT	Primary Hyperparathyroidism
2 ^o HPT	Secondary Hyperparathyroidism

Chapter 1

Introduction

1.1 Bone

Bone is a dynamic connective tissue consisting of an inorganic and organic phase. Approximately 60% of bone tissue is inorganic matter, 8-10% is water and the remainder is organic matter [1]. The inorganic phase of bone tissue is an impure form of hydroxyapatite ($\text{Ca}_{10}[\text{PO}_4]_6[\text{OH}]_2$) – a naturally occurring calcium phosphate. The organic phase is predominately made up of type I collagen and a variety of non-collagenous proteins and cells [2]. The structure of bone and its composition reflect a balance between its two major functions; the provision of mechanical integrity for locomotion and protection, and involvement in the metabolic pathways associated with mineral homeostasis [1]. There are two types of bone within the body, cortical and trabecular bone. Cortical bone is dense and compact and forms the outer layer of bone whereas trabecular bone makes up the inner layer of bone and has a spongy, honeycomb-like structure (Figure 1.1) Cortical bone constitutes approximately 80% of our skeletal mass and the remaining 20% is that of trabecular bone [3].

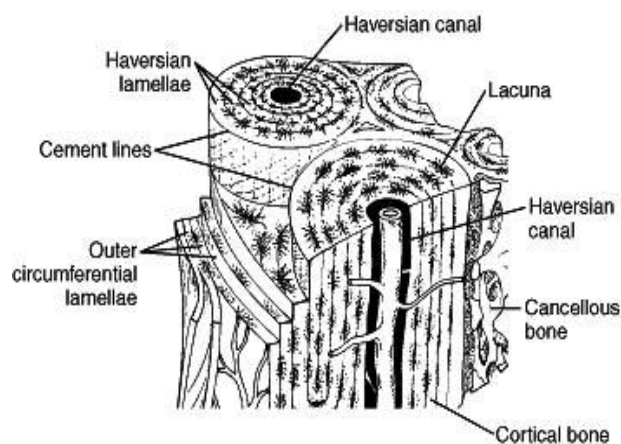


Figure 1.1 The microscopic structure of bone. Accessed 2010 (www.animusmedicalmindmaps.com).

1.1.1 Bone Remodelling

To maintain the structure-function relationship, bone is constantly broken down and rebuilt in a process known as bone remodelling and this process leads to the replacement of between 4 and 10% of bone annually in the adult human [1]. Bone remodelling is a coordinated process known as coupling in which there is localised removal of old bone,

(resorption), and the replacement with newly formed bone (formation). There are four types of cell involved in bone remodelling which are responsible for the production, maintenance and resorption of bone:

1. **Osteoblasts** are mononucleate cells that descend from mesenchymal stem cells in bone marrow [4] and are responsible for the production of the protein matrix of bone which is made up of type 1 collagen and several non-collagenous proteins. This protein matrix is known as osteoid which creates the template for mineralisation and the production of mature bone [1].
2. **Lining cells** are flat inactive osteoblasts that form a membrane at the surface of the bone tissue.
3. **Osteoclasts** are multinucleated cells that originate from monocytes and are responsible for the resorption of calcified bone and cartilage. During the process of bone resorption osteoclasts lie in small pits on the bone surface that are created by the digestion of underlying bone. The pits (or Howship's Lacunae) are formed in a sealing zone from the attachment of the cells plasma membrane to the underlying bone. A ruffled border secretes acid onto the lacunae thus acidifying and aiding dissolution of the mineralised bone matrix.
4. **Osteocytes** are also from the mesenchymal cell lineage and are the most abundant cellular component of bone. They are mature bone cells and their primary role is the maintenance of cellular activity and the regulation of bone turnover. Osteocytes are thought to be cells that control osteoblastic and osteoclastic activity within a basic multicellular unit (BMU), where bone remodelling occurs [5]. Osteocytes generate an inhibitory signal that is passed through their cell processes to osteoblasts for recruitment to enable bone formation [6].

Bone remodelling is complex, requiring the balanced interaction between osteoblasts and osteoclasts that are regulated by biochemical as well as mechanical factors on trabecular bone surfaces and in harversian systems [7]. The bone remodelling cycle of resorption and formation can be summarised in five phases (Figure1.2):

1. **Activation** begins as a result of mechanical or biochemical stimuli from quiescence. Under the influence of parathyroid hormone (PTH), calcitriol, or other bone marrow derived hormonal or cytokine signals osteocytes start to withdraw and swell. This action exposes the thin layer of unmineralised bone matrix or osteoid that encases the surface of bone. Newly activated epithelial or bone lining cells send chemical signals to pre-osteoclasts of the monocyte - macrophage lineage and osteoclast precursor cells are

recruited to the site and become fused to form differentiated, multinucleated osteoclasts [8].

2. **Resorption** is initiated by the newly formed or mature osteoclast that attaches to the bone surface. In order to remove bone, osteoclasts must become polarised and form a ruffled membrane which enables adherence to the bone surface. Hydrogen ions generated by carbonic anhydrase II are pumped across the ruffled membrane by a proton pump which dissolves bone. Enzymes are then released in order to degrade bone matrix [8].
3. **Reversal** represents the start of the repair process in which osteoclasts undergo apoptosis. Osteoclasts are replaced by mononucleated cells that smooth off the interior of the resorption pit and deposit a collagen poor binding layer that cements newly deposited bone to the old bone surface [1, 8].
4. **Formation** is a process whereby osteoblasts are attracted to the area by bone derived growth factors and chemical signals released by dead osteoclasts [8]. Type I collagen is then secreted in layers by the osteoblasts together with osteocalcin and other proteins to fill in the cavity forming new bone matrix.
5. **The resting phase** or quiescence or represents the end stage of the remodelling process. Following the matrix mineralisation osteoblasts flatten onto the new surface and then differentiate into bone lining cells. Some of the remaining osteoblasts become trapped in bone matrix to become osteocytes [8].

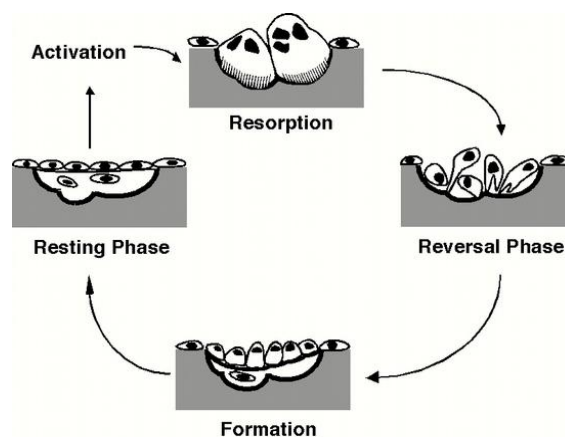


Figure 1.2 The bone remodelling cycle illustrating the cellular process of bone formation and resorption. Accessed 2010 (www.bioscience.org).

Remodelling allows bone to respond and adapt to stresses and mechanical loading such as physical exercise and under normal circumstances bone remodelling follows the sequence of resorption and formation and the resorption and formation rates are equally balanced [8], although rates of bone remodelling vary from site to site. The rate of

remodelling for trabecular bone is much higher than cortical bone throughout life; it is remodelled 5 – 10 times more rapidly than cortical bone [8]. Histologically bone is composed of individual BMU's [9] which are the coupled functional unit of osteoclasts and osteoblasts [10] and annually, the healthy adult may resorb and replace approximately 3 million BMUs, each taking 3 to 4 months from activation to completion [11]. The bone remodelling cycle is regulated by local and systemic factors. At the earliest phase of remodelling, osteoblastic marker runt-related transcription factor (Runx2) is necessary for progenitor cell differentiation along the osteoblast lineage [12]. During the sequence of cellular proliferation, Runx2 regulates the expression of genes encoding osteocalcin, VEGF, RANKL and sclerostin [13]. A large number of paracrine, autocrine, and endocrine factors affect osteoblast development and maturation. For example, bone morphogenetic proteins (BMP's), growth factors FGF and IGF, angiogenic factors endothelin-1, hormones such as parathyroid hormone (PTH) and prostaglandin agonists, all modulate osteoblast differentiation [14]. The fully differentiated osteoblast is characterised by its co-expression of alkaline phosphatase (ALP) and type I collagen (PINP), which are both essential for the synthesis of bone matrix and its subsequent mineralisation [15]. Mature osteoblasts also produce regulators of matrix mineralisation including osteocalcin, osteopontin (OPN) and osteonectin (ON) and RANKL which is necessary for osteoclast differentiation. When osteoblasts reach the end of their lifespan they are transformed into either osteocytes or lining cells. Specific molecules expressed by osteocytes include fibroblast growth factor-23 (FGF 23) and sclerostin, which control bone formation and phosphate metabolism [16]. Osteoclast differentiation is dominated by the RANKL/RANK/OPG pathway. This pathway is based on osteoblasts promoting osteoclast differentiation through the membranous secretion of RANKL and the binding of this factor to the membrane receptor RANK on osteoclast precursors [17]. The promotion of osteoclast differentiation by the receptor activator of nuclear factor kappa B ligand (RANKL) is inhibited by the decoy receptor osteoprotegerin (OPG), a glycoprotein and member of the tumour necrosis factor (TNF) receptor superfamily, which is also produced by osteoblasts [18]. Oestrogens increase OPG and decrease RANKL expression in osteoblasts, thus increasing bone formation. Postmenopausal bone loss is linked to reduced oestrogen levels resulting in increased resorption and subsequent bone loss [17].

1.1.2 Mineral homeostasis

Bone plays a vital role in human physiology. The word 'homeostasis' means steady-state where several ionic concentrations must be maintained at constant levels, one of which is calcium. Ninety nine percent of the body's calcium is contained in the skeleton [8] and the remaining 1% is found in the blood and soft tissues, contributing to normal nerve function,

muscle contraction, hormone secretion and enzyme activities such as blood clotting [19]. Approximately half the calcium in the bloodstream circulates as free, or ionized, calcium, and is available for use in these functions. The other half circulates while attached to proteins, and is less readily bioavailable [19]. In a healthy adult, calcium is maintained at a concentration of approximately 10mg/dL in blood serum. The mineral and organic composition of plasma, extracellular and intracellular fluid must be maintained within tight limits by the regulation and modulation of calciotropic hormones, including PTH, calcitonin and vitamin D [20, 21]. Calcium concentrations are maintained by a complex interrelationship between intestinal absorption, the renal retention of calcium, PTH secretion by the parathyroid gland, calcitonin secretion by the thyroid and osteoclastic activity (Figure 1.3) [8, 11]. Calcitonin is produced in the parafollicular or C-cells in the thyroid and it acts to reduce serum calcium opposing the effects of PTH [22, 23]. Parathyroid hormone maintains the levels of calcium in extracellular fluid by increasing the release of calcium from bone and osteoclast precursors are stimulated to differentiate into osteoclasts. Parathyroid hormone also increases the renal tubular resorption of calcium and promotes the synthesis of 1, 25 dihydroxyvitamin D₃ in the kidney; the principle effect of 1, 25 dihydroxyvitamin D₃ is to increase serum calcium levels by promoting intestinal absorption [24]. Vitamin D deficiency leads to a loss of mineralisation to the collagen matrix in bone, leading to rickets in children and osteomalacia in adults [25-27].

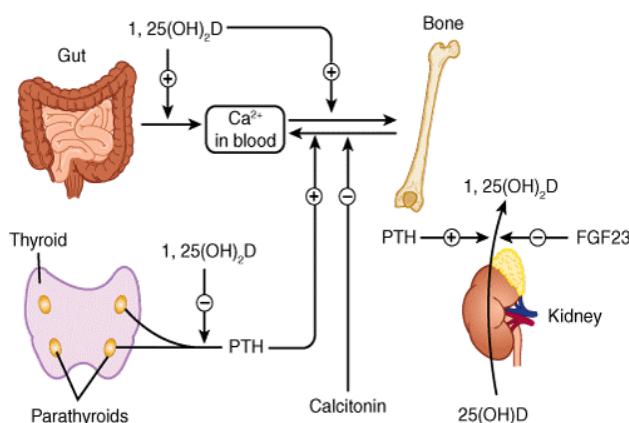


Figure 1.3 Calcium homeostasis. Calcium concentrations are maintained by a complex interrelationship between intestinal absorption, the renal retention of calcium, PTH secretion by the parathyroid gland, calcitonin secretion by the thyroid and osteoclastic activity. Accessed 2010 (www.renalmed.co.uk).

1.1.3 Skeletal structure and growth

The adult skeleton is comprised of 206 bones in total, which vary in size and shape and there are two main parts, the axial and the appendicular skeleton. At the moment of birth

we have approximately 275 bones that will later fuse to form the axial skeleton to which the appendicular skeleton is attached. The axial skeleton forms the central axis of the body and is responsible for maintaining the upright posture; it consists of the skull, the vertebral column, the ribs and the sternum. The appendicular skeleton refers to the remaining sections of the skeleton that are made up of long bones. Bones fall into five distinct classifications based on their shape: long, short, flat, irregular and sesamoid. Bone growth begins during early embryonic development between 6 – 7 weeks of gestation. During childhood and adolescence rapid skeletal growth occurs as linear and appositional growth [11]. Linear growth usually reaches its maximum at the latter half of the second decade [11]. Bone mass continues to increase by appositional growth, and peak bone mass is reached during the third decade of life at about the age of 35 for cortical bone [28] with trabecular bone mass peaking much earlier between 25 -30 years of age [29] (Figure 1.4). Peak bone mass is defined as the amount of bony tissue present at the end of skeletal maturation and is a major determinant of bone mass in later life [30-32]. With age, the density and internal architecture of bone starts to change. Age related bone loss in both men and women begins at about the age of 40 and continues throughout life. The remodelling process becomes imbalanced resulting in a decline in cortical thickness and trabecular density [33]. In cortical bone the haversian canals become widened leading to increased intracortical porosity and trabecular bone also comes under strain as the trabeculae become thinner resulting in perforation and a loss of connectivity [11]. Women lose approximately 35% of cortical bone and 50% of trabecular bone during a lifetime whereas men lose approximately two-thirds of this amount in their lifetime [1, 34]. The changes to bone throughout the skeletal structure are not uniform and the most notable change is seen within the vertebrae with the loss of both horizontal and vertical trabeculae [11].

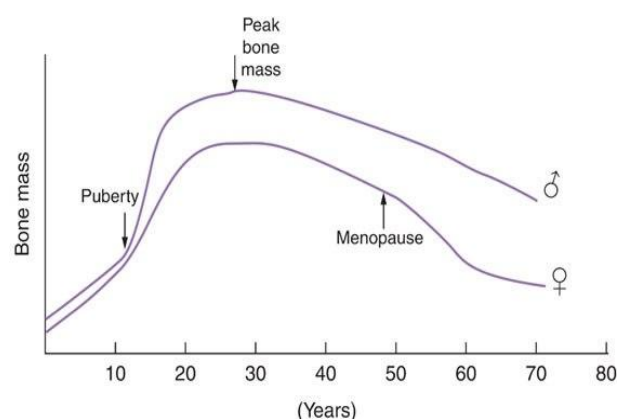


Figure 1.4 Lifetime changes in bone mass for men and women. Accessed 2010 (physrev.physiology.org).

1.2 Techniques for measuring bone mineral density (BMD)

There are a variety of methods that can be utilised for the measurement of BMD, each with advantages and disadvantages. Each technique varies in precision and differs substantially in methodology, clinical and research utility and availability [35]. Methods of quantifying BMD include dual energy x-ray absorptiometry (DXA), spinal and peripheral quantitative computed tomography (QCT/pQCT), and quantitative ultrasound (QUS). Spinal QCT is used to measure the volumetric BMD of trabecular bone and/or cortical bone that forms the exterior walls of the vertebrae [36]. Trabecular bone is much more metabolically active compared to cortical bone and is affected by age, disease and therapy-related changes earlier and to a greater degree than cortical bone. The advantage of QCT is that it is able to detect early changes in BMD in response to treatment or disease [37]. Peripheral QCT (pQCT) also uses CT to measure BMD at peripheral body sites such as the forearm and leg, and is useful for measuring bone strength [38].

1.2.1 Dual Energy X-ray Absorptiometry (DXA)

DXA imaging allows the 2-dimensional visualisation and analysis of bone and the surrounding soft tissue. During DXA analysis, an edge detection algorithm is used to determine the edges of the bone. The area of the bone is derived by summing the pixels within the bone edges, and the BMD is calculated from the mean BMD over all pixels that are identified as bone. Using the surrounding soft tissue adjacent to bone is an important part of the overall image analysis. It serves as a reference area of comparable thickness and composition. Dual energy x-ray absorptiometry uses an x-ray tube that emits x-rays at two different energies collimated to a narrow beam. When these pass through the subject, the intensity of the beam is registered by a detector situated directly opposite and the attenuation profiles of the two energy beams are recorded (Figure 1.5). Dual energy X-ray beams are produced by continuously switching the tube voltage between high and low values. The attenuation coefficient is dependent on the atomic number of the material/tissue being x-rayed and photon energy, and this allows the areal densities of bone mineral (hydroxyapatite) and soft tissue to be inferred [39]. Dual energy x-ray absorptiometry enables the measurement of BMD at clinically important sites such as the hip, lumbar spine and forearm. Bone mineral content (BMC) and bone area are calculated to provide areal BMD (g/cm^2). In addition, whole body composition scans can be performed using DXA, providing valuable information such as fat and lean percentages as well as BMC and total body mass. The wealth of information provided by whole body DXA scans has made them a useful tool in research. The advantages of DXA include short

scan times, minimal scanning preparation, low radiation dose and good measurement precision.



Figure 1.5 Image showing the mechanism of DXA. DXA uses an x-ray tube that emits x-rays at two different energies collimated to a narrow beam. When these pass through the subject, the intensity of the beam is registered by a detector situated directly opposite and the attenuation profiles of the two energy beams are recorded. Accessed 2012 (Isda.jsc.nasa.gov).

For many years it has been widely accepted that osteoporotic fractures are associated with increased morbidity and mortality. Advances in the field came with the ability to diagnose OP before fractures occur and with the development of effective treatments and the measurement of BMD played an essential role in both these developments [40]. Bone mineral density measurements are important for the evaluation of patients at risk of OP and for determining the appropriate use of anti-fracture treatment [41-43]. Dual energy X-ray absorptiometry scans are primarily used to scan areas of the central skeleton to measure BMD of the lumbar spine and hip [40]. Bone mineral density at the hip is the most reliable measurement for predicting hip fracture risk [44-46] and the spine is an important skeletal site for monitoring treatment effects [47-49]. It should be noted however that DXA may have the disadvantage of not being able to distinguish between OP and osteomalacia.

1.3 Osteoporosis

Osteoporosis is a term derived from the Greek language and directly translated means porous bone, “osteo” meaning bone and “poros” meaning pore [50]. The terminology

associated with OP was developed by German pathologists in the nineteenth century, initially to distinguish it from other conditions such as osteomalacia and osteitis fibrosa cystica [11]. In 1941, Albright et al defined OP pathologically ‘as a condition in which there is a lack of bone tissue, but that tissue which remains is fully calcified’ [51]. The current definition of OP originated with a Consensus Development Conference (CDC) in 1999 [52] and was given credibility by a World Health Organisation (WHO) Study Group in 1994 [53]. It defines OP as:

‘A progressive systemic skeletal disease characterised by low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture’ [42].

The WHO later suggested that low BMD and fracture be amalgamated and stratified into clear definitions (Table1.1). A T-Score is the number of standard deviations below the average for a young adult at peak bone density.

Table1.1 World Health Organisation classification of osteoporosis [53].

Definition	Criteria
Normal	BMD value of greater or equal value to 1 SD below the young adult mean value ($T \geq -1.0$)
Osteopenia (low bone density)	BMD value that lies between 1 and 2.5 SD below the young adult mean value ($T -1.0 - >2.5$)
Osteoporosis	BMD value greater than or equal to 2.5 SD below the young adult mean value ($T \leq -2.5$)
Established osteoporosis	BMD value greater than or equal to 2.5 SD below the young adult mean value ($T \leq -2.5$) with the presence of one or more fragility fractures.

T- Scores are calculated by taking the difference between a patient’s measured BMD and the mean BMD in healthy young adults, matched for gender and ethnic group, and expressing the difference relative to the young adult population standard deviation (SD):

$$\text{T-score} = \frac{\text{Measured BMD} - \text{young adult mean BMD}}{\text{Young adult population SD}}$$

Figure 1.6 T-score (WHO) [53].

Measurement of BMD also provides a Z-score which can be used to determine whether a loss of bone density is secondary to another disease or condition. The Z-score is not as widely used as the T-score however, it remains a useful concept as it expresses an

individual's risk of sustaining an osteoporotic fracture relative to their peers. Z-scores are similar to T-scores, however, instead of comparing the patient's BMD with the young adult mean, it is compared with the mean BMD expected for a healthy normal subject matched for age, gender and ethnic group:

$$\text{Z-score} = \frac{\text{Measured BMD} - \text{Age matched mean BMD}}{\text{Age matched population SD}}$$

Figure 1.7 Z-score (WHO) [53].

The important clinical use of BMD measurements is their ability to predict fracture risk. A recent advance in fracture risk prediction has led to the development of a diagnostic tool which provides a 10-year fracture risk probability called FRAX. FRAX integrates clinical risk factors (CRFs) and BMD at the femoral neck to calculate the 10-year probability of hip fracture and a major osteoporotic fracture (clinical spine, forearm, hip or shoulder fracture) [54]. An estimation of the 10-year fracture probability is calculated in men or women using age, body mass index (BMI), and CRFs. The CRFs assessed include a prior fragility fracture, parental history of hip fracture, current tobacco use, long-term use of glucocorticoids, rheumatoid arthritis, other causes of secondary OP and daily alcohol consumption. The 10-year risk of fracture can be calculated with or without femoral neck BMD in the model [55]. FRAX is incorporated into many national guidelines around the world, including those of Belgium, Canada, Japan, Netherlands, Poland, Sweden, Switzerland, UK (NOGG), and US (NOF).

1.4 Risk factors for osteoporosis

Osteoporosis is the most prevalent metabolic bone disease that affects an estimated 10 million Americans and 3 million people in the UK over the age of 50 [58,59]. Various risk factors are associated with the development of OP and are categorised as either modifiable or non-modifiable risk factors. The prevalence of OP increases markedly with age and after menopause and by the age of 60, approximately 15% of all women will have OP, with the incidence increasing to over 25% by the age of 80 [56]. Osteoporosis has great personal and economic burden. In Europe, disability due to OP is greater than that caused by cancers (with the exception of lung cancer) and is comparable with or greater than that resulting from a variety of chronic non-communicable diseases, such as rheumatoid arthritis [57].

Osteoporosis is often classified as either primary or secondary OP. A diagnosis of primary OP follows the exclusion of chronic disease as an identifiable cause and is associated

with the process of normal ageing. It is six times more common in women than men, occurring in women 15 to 20 years after menopause. The loss of bone is linked to oestrogen deficiency in women and a testosterone deficiency in men. There is a rapid loss of trabecular bone in primary OP resulting in the increased risk of vertebral and forearm fracture [58]. Secondary OP is used to define OP where there is a clearly identifiable cause such as disease or prolonged drug use, and it can arise at any age and affect men and women equally. It occurs in almost two-thirds of men, more than half of premenopausal and peri-menopausal women and about one-fifth of postmenopausal women [59].

1.4.1 Heritability

Previous twin and epidemiological studies have demonstrated that bone mass is under strong genetic control [60-65]. Inherited bone size, body composition and endocrine function may also explain genetic similarities however it is difficult to separate genetic and environmental roles in the determination of bone mass. One of the most important determinants of bone loss in women is oestrogen deficiency at menopause, and previous studies have indicated that age at menopause is genetically determined [66].

1.4.2 Gender

Gender plays an important part in bone mass development. It is widely accepted that men have a larger bone size than women and greater cortical mass [67, 68]. Puberty also plays an important role in determining gender differences in bone size and mass but this is not fully understood [69]. Before puberty, in both sexes, linear and appositional skeletal growth increases progressively. Boys enter puberty 2-years later than females and as a result can acquire greater bone length prior to puberty [70, 71]. A study by Specker et al showed that physical activity combined with a high calcium intake increased both bone circumference and cortical thickness, suggesting that gender-related differences in bone size are to some extent related to variation in physical activity, dietary factors or the effect of muscle mass on bone geometry [72]. In conclusion, males do have greater bone density and larger bone geometry but these differences are only partly explained by gender differences in body size [69].

1.4.3 Ethnicity

It is well documented that there is great variation in both skeletal size and mass between racial groups [73-76]. These may be attributed to genetic factors although there are environmental factors such as diet and physical activity that may account for the wide variations. Black people have larger denser bones than whites, while the Chinese have a smaller skeletal mass [11]. Dietary factors may play a greater role in defining racial variation. For example, the correction of a low calcium diet in the Japanese population has led to an increase in average height [11].

1.4.4 Hormonal influences

Women have a lower bone mass than men, and this difference increases following the menopause. The factors that influence skeletal development and determine gender differences have been assumed to be related primarily to sex steroid action. Sex steroids are synthesised in response to signals from the brain. It is this stimulus from the hypothalamus to the pituitary gland which releases hormones that target the reproductive organs [77]. Luteinising hormone (LH) and follicle-stimulating hormone (FSH) stimulate the synthesis of progesterone and oestrogens in the female ovaries and testosterone in the male testes [77-79]. With the onset of puberty, hormones contribute to gender differences and the greater bone mass in men [70]. Studies have shown that gonadal hormones can increase bone formation [80] particularly in cortical areas, and the absence of androgen action has been related to lower bone mass and smaller size [81]. Testosterones are known to influence weight and muscle mass and therefore also indirectly affect skeletal development. Oestrogens slow the rate of bone remodelling and help to maintain a balance between bone formation and resorption by increasing the release of osteoclast and osteoblast progenitor cells in the bone marrow [82-84]. Conversely, oestrogen deficiency causes an increase in bone remodelling where osteoclastic activity is increased coupled with a reduction in osteoblastic activity, resulting in increased resorption and reduced formation leading to bone loss [85].

1.5 Modifiable risk factors for osteoporosis

1.5.1 Dietary factors

The contribution of dietary calcium to bone mass has been debated but it has been shown that a high lifelong calcium intake is associated with a higher bone mass [86]. Calcium and vitamin D are the major determinants of calcium balance which in turn reflects

calcium intake, absorption and excretion. Adequate calcium intake is important throughout life in order to maintain bone density and if calcium intake is suboptimal peak bone mass may not be attained [1]. Dietary factors such as protein and vitamin K also play an important role in bone status along with other essential nutrients and trace elements such as magnesium, zinc and copper. The effects of protein on bone have been contradictory with studies indicating that protein increases renal excretion of calcium [87-89] while others have shown protein slows down age-related bone loss [90-92]. It has been suggested that vitamin K has a role in bone metabolism, and inadequate vitamin K intake has been thought to increase the risk of osteoporotic fracture [93]. Three vitamin K-dependent proteins, osteocalcin, matrix Gla protein, and protein S, are found in bone and osteocalcin is the most abundant [93]. The exact role of vitamin K is unclear, however it has been shown to have a protective effect on bone, increasing osteoblastogenesis and decreasing osteoclastogenesis, thereby increasing bone formation and decreasing bone resorption [93].

1.5.2 Physical inactivity

The role of regulation of bone structure by physical activity has been shown to begin as early as 5 -7 weeks of prenatal life [94, 95]. Environmental influences during childhood and puberty have been shown to benefit bone mineral development. Physical activity in childhood stimulates the bone remodelling process and expands bone size to produce larger and stronger bones [96, 97]. Weight bearing exercise is believed to increase bone density by direct stimulation of the bone remodelling process, while a lack of weight bearing activity and immobilisation due to bed rest, paraplegia and also during space flight is known to produce rapid bone loss in humans [98-100]. It has been reported that the initial bone loss as a result of immobilisation is approximately 1% per week and up to 4% of bone mass can be lost per month during initial phases of bed rest [11, 101]. Bone remineralisation begins again following the re-introduction of weight bearing exercise following long periods of inactivity, however this process is much slower and often incomplete [102]. Studies have shown the beneficial effect of weight bearing activity on bone [103-106] suggesting that weight bearing exercise is an important factor in the regulation and maintenance of bone mass.

1.5.3 Body mass

Low weight, or a low BMI of $<20\text{kg/m}^2$ is a well-documented risk factor for future fracture, whereas a high BMI appears to be protective [107-113]. A BMI of 20kg/m^2 predicts a two-fold increased risk of fracture compared to people with a BMI of 25kg/m^2 [114].

1.6 Secondary causes of osteoporosis

Hypogonadism is a well-established cause of OP in males. Conditions that may lead to hypogonadal OP in men are hemochromatosis and primary testicular failure [98]. Both cortical and trabecular bone are lost in hypogonadal osteoporosis and although the pathogenesis of this is unclear, the bone loss may be a result of androgen or oestrogen deficiency, low plasma 1,25-dihydroxyvitamin D leading to poor calcium absorption and reduced calcitonin levels [98].

Glucocorticoid-induced osteoporosis (GIOP) is the most common form of drug-induced OP [115-118] and is due to a combination of increased bone resorption and decreased bone formation. Many chronic rheumatological disorders are treated with glucocorticoid therapy including rheumatoid arthritis (RA), polymyalgia, temporal arteritis and systemic lupus erythematosus (SLE), however it is also used to treat asthma, chronic obstructive pulmonary disease (COPD), inflammatory bowel disease (IBD), and a variety of skin disorders [119-124].

Chronic gastrointestinal (GI) diseases leading to impaired GI absorption (malabsorption) are a risk factor for OP and osteomalacia [11, 125]. The most common causes of malabsorption are coeliac disease, blind loop syndrome as a result of intestinal obstruction and gastrectomy [11]. Coeliac disease is a genetically-determined chronic inflammatory intestinal disease caused by gluten intolerance [126] and has been found in approximately 3% of patients suffering from OP [127-129]. The pathogenesis of OP associated with coeliac disease is not well understood and it is unclear whether bone loss results from inadequate bone formation or from excessive bone resorption [127]. However, the most important mechanism of BMD loss is most likely due to initial calcium malabsorption caused by villous atrophy and by coexisting vitamin D deficiency [130, 131]. This leads to secondary hyperparathyroidism, increased bone resorption, and increased but inadequate bone formation [132-135].

Diabetes mellitus (DM) is associated with primary OP [136, 137]. The initial onset of type 1 DM often occurs at a young age, when bone mass is still being accrued [138]. In individuals with poorly controlled type 1 DM, poor nutrition, excessive loss of calcium in the urine and ketoacidosis may all lead to bone loss and a failure to attain peak bone mass [136, 139].

Primary hyperparathyroidism (1° HPT) is a common disease in postmenopausal women [140, 141]. Parathyroid hormone is secreted by the parathyroid gland and is involved in bone metabolism by regulating the level of calcium in the blood, release of calcium from

bone, absorption of calcium from the intestine, and excretion of calcium in the urine [142]. The overproduction of PTH in 1^o HPT leads to hypocalcaemia and other metabolic changes and increased bone turnover, with a loss of bone more notable in cortical than trabecular bone [143]. Secondary hyperparathyroidism (2^o HTP) often occurs in older people and is a physiological adaption to hypercalcaemia and is often a result of intestinal disease leading to malabsorption [144].

Thyrotoxicosis is a metabolic syndrome associated with elevated levels of the thyroid hormones, thyroxin (T4) or triiodothyronine (T3). Elevated levels of thyroid hormone (hyperthyroidism) are associated with an increase in bone formation and resorption, although resorption usually exceeds formation resulting in bone loss, OP and an increased risk of fracture [107, 145].

Chronic kidney disease (CKD) is a risk factor for secondary OP. Renal osteodystrophy or chronic kidney disease-mineral and bone disorder (CKD-MBD) [146] is a term applied to bone pathology to describe abnormalities in bone mineralisation and metabolism that occur in chronic kidney disease (CKD) [147], and OP is frequently associated with CKD stages 3-5 [148, 149]. CKD-MBD is the result of hyperparathyroidism secondary to hyperphosphatemia combined with hypocalcaemia, both of which are due to decreased excretion of phosphate by diseased or damaged kidneys. Low activated vitamin D₃ levels are a result of the damaged kidneys inability to convert vitamin D₃ into its active form, calcitriol, and result in further hypocalcaemia [150].

1.7 Fractures in osteoporosis

The significant clinical outcome in OP is the increased likelihood of fracture. Fragility fractures at the spine, hip and forearm are the most typical sites, although fractures of the ribs, humerus and pelvis are not uncommon. The annual incidence of osteoporotic fractures (including recurrent ones) is 180,000 in England and Wales [151]. The combined lifetime risk for hip, forearm and vertebral fractures coming to clinical attention is around 40%, equivalent to the risk for CV disease [152]. Fractures result in severe pain and disability and the cost of osteoporotic fractures to the National Health Service (NHS) each year is over £1.8 billion [153] and with an ageing population, it is estimated that the number of osteoporotic fractures over the next 50 years will double in Europe [154]. There is considerable morbidity after hip fracture, with 25-50% of patients becoming more dependent and many needing residential or nursing care [155]. Mortality rates are approximately 10-20% after hip fracture; with an estimated 14,000 people dying each year in the UK following an osteoporotic hip fracture [156].

1.7.1 Vertebral fracture

Vertebral fractures affect approximately 20% of postmenopausal women [157, 158] and are the most common complication of OP. Vertebral fractures are associated with significant pain, morbidity, excess mortality and health and social service expenditure resulting from long-term disability. There is also an increased risk of further fractures in patients who have already sustained fractures [159, 160]. It is increasingly difficult to quantify the incidence of vertebral fracture as only a third of patients come to medical attention after fracture [161]. However, The European Prospective Osteoporosis Study (EPOS) assessed vertebral fracture incidence in 3174 men and 3614 women over a 3 year period and reported vertebral fracture incidence increases markedly with age in both men and women [162]. Vertebral fractures commonly occur in the upper lumbar or lower thoracic vertebrae [161] and if symptomatic, typically present with the sudden onset of severe pain [163], and there is often an associated loss of height and thoracic kyphosis. One of the most important factors impairing quality of life is back pain. The EVOS study [159] demonstrated that patients with three or more vertebral deformities were almost twice as likely to report back pain in the previous year, compared with subjects without deformity.

1.7.2 Hip fracture

The most severe osteoporotic fracture is that of the hip which may occur as a result from a fall from standing, but may also occur spontaneously [98]. Hip fractures present with severe pain and almost always require hospitalisation and surgical intervention [98], and are more common in the elderly, mostly occurring at around 80 years of age and they are four times more common in women [164, 165]. It is estimated that by the year 2016, there will be around 117,000 hip fractures a year in the UK [164]. In addition to the role of advancing age on the occurrence of hip fractures, both skeletal and non-skeletal risk factors have been identified. Low BMD is considered to be the major risk factor for hip fractures [107, 166], with half of all elderly women presenting with a hip fracture having OP [44].

Hip fractures are associated with increased mortality rates [167] which may be linked to complications following the fracture, such as pulmonary embolism (PE), infections and heart failure [168-170]. In addition to high mortality rates, hip fractures also contribute to OP-associated disabilities such as pressure sores, bronchopneumonia and urinary tract infections [98]. Impaired mobility is one of the primary long-term outcomes following hip fracture and despite rehabilitation many patients fail to regain their pre-fracture ambulatory or functional status [171]. The risk of fracture increases with age in both men and women,

however, the rate of hip fracture is higher in women than in men [43] although clinical trials and research on hip fracture outcomes have mainly focused on Caucasian women [172].

1.7.3 Forearm fracture

Distal forearm fractures account for approximately 15% of all fractures in adults [173]. The most common forearm fracture is the Colles' fracture, and nearly always follows a fall on an outstretched arm. This fracture was first described by Irish surgeon and anatomist, Abraham Colles, in 1814 [174]. Colles' fractures are common in women between the ages of 50-70 years of age and often require surgical intervention. It is characterised by a fracture of the distal radius in the forearm with dorsal (posterior) displacement of the wrist and hand. Complications following Colles' fracture include pain and tenderness, vascular instability, swelling and stiffness, known collectively as algodystrophy [175]. Approximately 95% of distal forearm fractures are the consequence of a fall [176]. Risk factors for wrist fracture include low BMD, a history of two or more falls in the preceding year, and a previous fracture after the age of 50. Poor cognitive status increases the risk for wrist fracture in women over the age of 75 years [177]. The incidence of Colles' fractures peaks in winter, more so than that of hip fractures, and is closely related to falls outdoors during icy weather conditions.

1.8 Therapy for Osteoporosis

There are many treatments available that can help to maintain or increase bone density therefore reducing the risk of fracture. For those already affected by OP, the prompt diagnosis of bone loss and assessment of fracture risk are essential in order to establish the correct therapy available, to prevent further loss of bone and increase bone density.

1.8.1 Bisphosphonates

Bisphosphonates (BPs) are a class of drug known as anti-resorptive agents and have been an established treatment for bone loss, OP and hypercalcaemia in addition to metastatic bone disease for many years. Interest in the clinical application of BPs dates back to the 1960's when they were first investigated for use in disorders of bone metabolism [178]. Early in-vitro laboratory studies by Fleisch et al found that blood plasma and urine contained a compound that inhibited calcium phosphate precipitation [179]. This activity was in part due to inorganic pyrophosphate, a substance not described at the time. Further studies found that calcium phosphate dissolution was inhibited by pyrophosphate

in-vitro. However, in-vivo pyrophosphate prevented ectopic calcification but had no effect on normal mineralisation or bone resorption [180, 181]. It was suggested that the lack of activity on bone mineralisation was due to the local destruction of pyrophosphate by phosphatases. It was then discovered that analogs of pyrophosphate were not destroyed enzymatically, and from here the BP was developed [180, 181]. Bisphosphonates are chemically stable analogues of inorganic pyrophosphate and are compounds characterised by two distinct C–P bonds (Figure 1.8). As a chemical structure all BPs share a common PCP backbone which allows a great number of possible variations, mostly by changing the two lateral chains on the carbon (Figure 1.8) which can lead to extensive alterations in their physicochemical, biological, therapeutic, and toxicological characteristics [182].

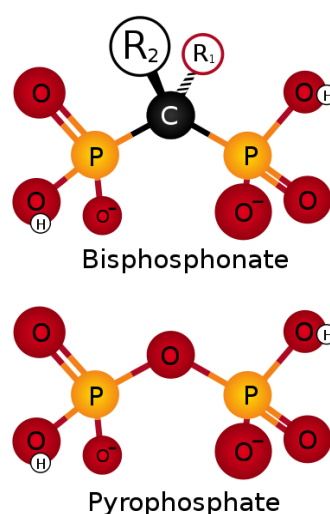


Figure 1.8 The chemical structures of bisphosphonate and pyrophosphate. Accessed 2012 (en.wikipedia.org).

The long R_2 side-chain (Figure 1.8) determines the chemical properties, the mode of action and the strength of the BP whereas the shorter R_1 side-chain mainly influences chemical properties and pharmacokinetics [183]. Most of the BPs, like pyrophosphate, inhibit the precipitation of calcium phosphate even at very low concentrations [180] and also slow down the dissolution of calcium crystals [182]. The largest store of calcium in the body is found in bone tissue and BPs act almost exclusively on bone when administered at physiological doses due to their affinity to calcium [184]. Studies have shown that 1 to 2 thirds of administered BP becomes incorporated into the skeleton and the remaining is excreted in the urine within the first few hours after administration [185]. Retention is affected in patients with high bone turnover and is also influenced by renal function [185].

There are two classes of BP; nitrogen containing and non-nitrogen containing and the two classes work differently at inhibiting osteoclastic action [186]. Nitrogen containing BPs act

on bone metabolism by binding and blocking the enzyme farnesyl diphosphate synthase in the mevalonate pathway [187], preventing the formation of metabolites that are essential for connecting some proteins to the cell membrane; a process known as prenylation. Inhibition of protein prenylation affects many proteins found in the osteoclast. These proteins can affect osteoclastogenesis, cell survival, and cytoskeletal dynamics [188]. The cytoskeleton is vital for maintaining the ruffled border that is required for contact between a resorbing osteoclast and a bone surface. Non-nitrogen containing BPs are metabolised in the cell to compounds that replace the terminal pyrophosphate moiety of adenosine triphosphate (ATP), forming a non-functional molecule that competes with ATP in the cell metabolism. As a result the osteoclast initiates apoptosis, leading to an overall decrease in the breakdown of bone [183].

Bisphosphonates are widely used in the management of postmenopausal OP, due to their efficacy in reducing the risk of fractures [189-191]. In postmenopausal OP, BPs reduce bone turnover markers, increase BMD at the lumbar spine and hip, and reduce the risk of vertebral and non-vertebral fractures [192-194]. Bisphosphonates are also used for the treatment of GIOP and male OP. A variety of BP treatments are available to treat OP as either oral or intravenous preparations but the most commonly used are oral BPs. Etidronate was the first to be approved for OP [194, 195], followed by alendronate [196, 197], risedronate [195], and ibandronate [198] and most recently Intra-venous (IV) zoledronate [199]. These BPs modestly increase BMD by 4% – 12% reducing fracture risk at the spine by 30-70% and also to varying degrees at other skeletal sites in postmenopausal women [195].

Oral BPs are not without side effects. Nitrogen containing BP preparations can cause gastric upset and inflammation and erosions of the oesophagus. Intravenous BPs can give fever and flu-like symptoms after the first infusion, which is thought to occur because of their potential to activate human gamma delta ($\gamma\delta$) T cells [200]. When administered intravenously for the treatment of cancer and OP, BPs have been associated with osteonecrosis of the jaw (ONJ) and some rarer side effects including atrial fibrillation and inflammatory eye disease. Current estimates of ONJ related to oral BP therapy for osteoporosis are between 1 in 10,000 to 1 in 100,000 patients per year. The incidence of ONJ in patients with cancer, who typically receive high doses of IV BP has been estimated to be 1 to 10 per 100 patients [201]. Recent reports have suggested a link between BP use and the development of atypical fractures of the femur as this is thought to be due to the long term over suppression of bone turnover leading to impaired bone remodelling and the accumulation of microdamage in bone, resulting in increased skeletal

fragility [202, 203]. Severe joint and muscle pain has also been reported as a side effect of BP treatment [204].

1.8.2 Denosumab

Denosumab is a fully human monoclonal antibody to the receptor activator of nuclear factor kappaB ligand (RANKL), a member of the tumour necrosis factor (TNF) super family of ligands and receptors essential for the function of bone-resorbing osteoclasts [205]. RANKL interacts with a receptor (RANK) on both osteoclast precursors and osteoclasts. The RANKL/RANK interaction results in activation, migration, differentiation, and fusion of hematopoietic cells of the osteoclast lineage to begin the process of bone resorption [205, 206]. Denosumab acts by blocking the binding of RANKL to RANK, reducing the formation, function, and survival of osteoclasts, which results in decreased bone resorption and increased bone density [205, 206]. In 2010, denosumab was approved for post-menopausal OP by the FDA [207] as it has been shown to improve low BMD in postmenopausal women [208-211]. Like BP therapy denosumab also has significant side effects including infections, ONJ and femoral fragility fractures.

1.8.3 Selective Oestrogen Receptor Modulators (SERMs)

Selective oestrogen receptor modulator (SERMs) is the name given to a class of compounds which are tissue-specific. A characteristic that distinguishes these substances from pure receptor agonists and antagonists is that their action is different in various tissues, thereby granting the possibility to selectively inhibit or stimulate oestrogen-like action in specific tissues [212]. SERMs block oestrogen receptors in breast tissue, but act as a pro-oestrogen in bone [11]. Raloxifene (Evista) is FDA approved for the prevention and treatment of OP in postmenopausal women. SERMs are considered good replacements for HRT, providing the benefits of oestrogen while avoiding the hormone's potential side effects such as an increased risk of breast cancer and deep vein thrombosis [213-215]. Raloxifene acts like oestrogen on bone, maintaining bone density and reducing fracture risk [216].

1.8.4 Hormone Replacement Therapy (HRT)

Hormone replacement therapy uses oestrogens, either alone or in combination with progesterone for the management of menopausal symptoms, and have also been shown to offer good protection for bone by reducing bone turnover and therefore reducing bone loss [217]. It has been suggested that HRT has other protective effects; protecting against

heart disease, maintaining cognitive function and reducing the risk of developing Alzheimer's disease [218-221]. In contrast to the beneficial effect of HRT on bone, and its protective role on other diseases, studies have reported health risks associated with HRT therapy including an increased risk of endometrial cancer in women who have not had a hysterectomy [213, 214] and some oestrogen-progestogen combinations may be associated with a small increased risk of breast cancer [222]. Due to the risks associated with long term HRT, it is not generally used as a first choice treatment for the long-term prevention OP. However, in postmenopausal women under the age of 60, who have a high fracture risk, HRT may be considered provided that the benefit, in terms of reducing fracture risk, outweighs any adverse risks for the individual. Hormone replacement therapy is not considered suitable for women who have risk factors for breast cancer, heart disease, stroke or blood clots [217].

1.8.5 Tibolone

Tibolone is a synthetic steroid hormone drug, which is fairly non-selective in its binding profile, acting as an agonist mainly at oestrogen receptors. It is used mainly for the treatment of endometriosis as well as HRT in postmenopausal women and retains a licensed indication for the treatment of OP.

1.8.6 Teriparatide and PTH 1-84

Parathyroid hormone 1-34 (teriparatide, PTH-34) and PTH 1-84 belong to a class of drugs called anabolic agents [223]. Parathyroid hormone (PTH) is an 84-amino acid polypeptide secreted by the parathyroid glands in response to small changes in serum calcium [224]. Teriparatide is a recombinant PTH and is used to treat OP by stimulation of bone formation [223-225]. The mechanism of teriparatide is to stimulate new bone formation on inactive bone surfaces, while stimulating bone turnover by the classic remodelling cycle involving both osteoclastic resorption and osteoblastic reformation [224]. Teriparatide improves skeletal architecture; bone is gained, which is recognised as increased trabecular thickness and connectivity [226, 227].

1.8.7 Calcium and vitamin D supplements

Calcium and vitamin D supplements are frequently given to the elderly and patients with osteoporosis. However they are mainly used as an adjunct therapy for those already on treatments for OP. Calcium plays a vital role in the regulation of various cells in the peripheral and central nervous systems, muscle and endocrine glands [228]. The

regulation of calcium homeostasis is aimed at maintaining extracellular calcium concentration and balance as constant as possible in addition to protecting against calcium deficiency or overload [228, 229]. It should be noted however, that there is an increased risk of renal calculi and of cardiovascular disease with calcium and vitamin D supplements. Intestinal calcium absorption declines with age in both men and women, and calcium deficiency may play a contributory role in the development of low bone density [230-232] and an increased risk of fracture.

Vitamin D activity is essential for the normal development of the skeleton. It is a steroid vitamin which encourages the absorption and metabolism of calcium and phosphorous. The natural form of vitamin D is cholecalciferol or vitamin D₃, which is synthesised in the skin by the direct action of ultraviolet (UV) light on 7-dehydrocholesterol [11]. The elderly are at high risk of vitamin D deficiency, especially the institutionalised, because of poor dietary intake and decreased exposure to sunlight [233, 234]. The 'European guidance for the diagnosis and management of OP in postmenopausal women' suggests that where there is a high prevalence of calcium and vitamin D deficiency in the elderly, combined calcium and vitamin D supplements in a daily dose of 500 – 1200mg and 400-800 IU respectively should be prescribed [235], particularly in those that are already receiving bone therapy.

1.9 Cardiovascular disease

Cardiovascular disease refers to any disease that affects the cardiovascular (CV) system, such as cardiac disease, vascular diseases of the brain and kidney, and peripheral arterial disease [236, 237]. The causes of CVD are diverse however atherosclerosis and/or hypertension are the most common causes. Cardiovascular disease usually affects older adults, and with age come a number of physiological and morphological changes that alter CV function and lead to an increased risk of CVD even in healthy asymptomatic individuals [238]. Although CVD is one of the leading causes of worldwide mortality CV associated mortality rates have declined in many high-income countries since the 1970s [239]. However, at the same time CV deaths and disease have increased at a fast rate in low and middle-income countries [240]. There is increased emphasis on preventing atherosclerosis by modifying risk factors, such as healthy eating, exercise, and smoking cessation. The WHO estimates that there will be approximately 20 million CVD related deaths in 2015, accounting for 30% of all deaths worldwide [241].

1.9.1 Cardiovascular risk factors

The majority of CVD is caused by risk factors that can be controlled, treated or modified, such as high blood pressure, cholesterol, obesity, tobacco use, physical inactivity and diabetes. However, there are also some major CVD risk factors that cannot be controlled.

1.9.2 Hypertension

There are at least 970 million people worldwide who have hypertension, with approximately 330 million people in the developed world and 640 million in the developing world documented as having hypertension [242]. The WHO rates hypertension as one of the most important causes of premature death worldwide and the problem is growing. In 2025 it is estimated there will be 1.56 billion adults living with high blood pressure [242]. Hypertension increases the risk for many CV diseases, including stroke, coronary artery disease, heart failure, and peripheral vascular disease [243]. Coronary disease in men and stroke in women are the principal CV events noted after hypertension onset, as reported by the Framingham Heart Study [244].

1.9.3 Smoking

Smoking is estimated to cause nearly 10% of CV related events, and is the second leading cause of CVD, after hypertension [245]. Approximately 6 million people die from the effects of tobacco use or as a result of passive smoking, accounting for 6 % of female and 12 % of male deaths worldwide, annually. By 2030 tobacco-related deaths are projected to increase to more than 8 million deaths a year [245]. An association between smoking and vascular disease has been recognised for many years [246] and is responsible for approximately 50% of acute myocardial infarctions (AMI) in young and middle-aged individuals [247] and a significant risk for coronary and peripheral artery disease [248, 249], abdominal aortic aneurysm and stroke [250, 251].

1.9.4 Diabetes

The most prevalent form of diabetes mellitus (DM) is type 2, and typically occurs in later life. The underlying metabolic causes of type 2 DM are the combination of impairment in insulin-mediated glucose disposal (insulin resistance) and defective secretion of insulin by pancreatic b-cells [252]. Insulin resistance develops as a result of obesity and physical inactivity, acting on the potential for genetic susceptibility [253, 254]. Insulin resistance precedes the onset of type 2 DM and is commonly accompanied by other CV risk factors including dyslipidaemia, hypertension, and prothrombotic factors [255, 256]. The collective

incidence of these risk factors is known as metabolic syndrome and many patients with metabolic syndrome present with impaired fasting glucose [257], even in subclinical DM [258]. It is the risk factors that constitute metabolic syndrome that contribute independently to CVD risk [252].

1.9.5 Physical inactivity

The increasing urbanisation and mechanisation of the world has resulted in a reduction in the levels of physical activity. The WHO suggests that more than 60% of the global population is not sufficiently active [259] and approximately 3.2 million deaths each year are attributable to a sedentary lifestyle [260]. Research has shown that all individuals can benefit from regular physical activity, whether they participate in vigorous exercise or moderate health-enhancing exercise [261, 262]. Mobility and function can be improved even in the most frail and elderly adults [263]. Individuals who are physically more active have lower rates of all-cause mortality [264], most likely due to a decrease in chronic diseases, including coronary artery disease (CAD). This low rate may result from an improvement in CV risk factors, enhanced fibrinolysis, improved endothelial function, physical activity and reduced CV risk [264].

1.9.6 Poor diet

Poor diet and physical inactivity, resulting in increased weight and obesity, are the most common risk factors for heart disease. The role of diet and nutrition is influenced by a range of social, cultural, economic and physiological factors, including the availability and cost of food. Diet is one of the modifiable risk factors that will impact all other CV risk factors [265]. Hypercholesterolemia, a known precursor for CVD, is caused by saturated fatty acid (SFA) and trans fatty acid (TFA) found in animal products and oils that have been hydrogenated to turn them into semi-hard fats. In terms of nutrition, a diet high in SFA and TFA has been associated with CHD incidence [266]. High sodium intake is also a major risk factor for hypertension and subsequent CVD. During the past century, the evidence for the risks to human health by excess salt consumption is undeniable with causal associations between dietary salt intake and blood pressure being established through experimental, epidemiological, migration, and intervention studies [267]. It has been estimated that a reduction in dietary intake of sodium by approximately 1g of sodium a day, about 3g of salt, would lead to a 50% reduction in the number of people needing treatment for hypertension. The same decrease would lead to a 22% drop in the number of deaths resulting from strokes and a 16% fall in the number of deaths from CHD [260].

1.9.7 Hypercholesterolemia

Hypercholesterolemia is a term used to describe the presence of high levels of cholesterol in the blood [268]. Lipoproteins are a biochemical assembly that contains proteins and lipids and classified by their density; very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) [269]. All the lipoproteins carry cholesterol, but elevated levels of the lipoproteins other than HDL (termed non-HDL cholesterol), particularly LDL-cholesterol are associated with an increased risk of atherosclerosis and CHD [270]. Hypercholesterolemia is attributed to a combination of environmental and genetic factors [271] including obesity and dietary choices and genetic contributions are usually due to the additive effects of multiple genes, though occasionally may be due to a single gene defect such as in the case of familial hypercholesterolemia [271]. Research has shown a strong correlation between hypercholesterolemia and the risk of CAD and coronary mortality [272-274].

1.9.8 Obesity

Obesity is a medical condition in which excess body fat has accumulated to the extent that it may have a serious adverse effect on health, leading to an increase in chronic disease and mortality [275, 276]. The worldwide epidemic of obesity is primarily due to an imbalance between physical activity and dietary energy intake. A sedentary lifestyle coupled with an unhealthy diet resulting in obesity markedly increases the risk of CVD [277]. Serious complications to health as a result of obesity include type 2 diabetes [278], cancer and non-alcoholic fatty liver disease [278, 279]. Just as importantly, obesity leads to a high prevalence of CVD including ischemic heart disease (IHD) [280], angina and MI, congestive heart failure (CHF) [276], deep vein thrombosis (DVT) and PE [281]. Systemic hypertension, pulmonary hypertension (left ventricular failure, chronic hypoxia), and CHD all occur with disproportionately high frequency in obese individuals and may cause or contribute to alterations in cardiac structure and function [282, 283]. The risk of sudden cardiac death is also increased in obesity. The Framingham Study [283], reported that the annual sudden cardiac mortality rate in obese men and women was estimated to be 40 times higher than the rate of unexplained cardiac arrest in a matched non-obese population [284, 285].

1.10 Atherosclerosis

Atherosclerosis is the most frequent underlying cause of CHD, carotid artery disease (CAD) and peripheral artery disease (PAD) [286]. Inflammation plays a major role in all

phases of atherosclerosis [286, 287]. It is caused by a chronic inflammatory response in the walls of the arteries and is caused by the accumulation of macrophages and promoted by LDL, plasma proteins that carry cholesterol and triglycerides without adequate removal of fats and cholesterol from the macrophages by functional HDL [288]. Atherosclerotic lesions are known as atheroma [289] and the atheromatous plaque has three distinct components (Figure 1.9):

1. The atheroma, which is the nodular accumulation of a soft, flaky, yellowish material at the centre of large plaques, composed of macrophages nearest to the lumen of the artery.
2. Underlying areas of cholesterol crystals.
3. Calcification at the outer base of older/more advanced lesions.

Depending upon their composition, plaques can be classified into two types [290]:

1. **Stable and low risk plaques.** These are relatively benign and less prone to thrombotic complications. Clinical symptoms present when it is large enough to compromise the lumen of a coronary artery, resulting in myocardial ischemia.
2. **Vulnerable, unstable or high risk plaque.** This is the dangerous type of plaque which is liable to complications, namely rupture, tear, erosion and production of coronary thrombosis.

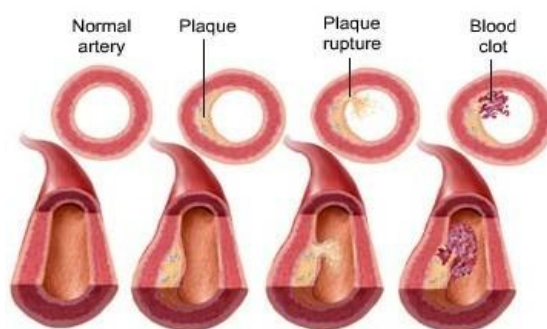


Figure 1.9 Diagram showing the progression and severity of atherosclerosis within the arterial lumen. Accessed 2011 (anatomyandphysiologyi.com).

1.10.1 Pathophysiology of atherosclerosis

Atherogenesis is a term to describe the formation of atheromatous lesions in arterial walls [291], and is characterised by a remodelling of arteries involving the sub-endothelial accumulation of atheromatous plaques. Early atherogenesis is characterised by the adherence of blood circulating monocytes to the endothelium of the artery, followed by their migration to the sub-endothelial space, and further activation into monocyte-derived

macrophages [292]. This process is caused by oxidized LDL particles from plasma invading the endothelium. A complex set of biochemical reactions regulate the oxidation of LDL, involving enzymes such as lysophosphatidic acid LpA-LpA2 and free radicals in the endothelium [292]. Lysophosphatidic acid is a bioactive phospholipid produced by activated platelets and formed during the oxidation of LDL. Accumulating evidence suggests that this lipid mediator may serve as an important risk factor for development of atherosclerosis and thrombosis [293-298]. Initial damage to the endothelium results in an inflammatory response where monocytes enter the artery wall from the bloodstream, with platelets adhering to the target area. This may be promoted by redox signalling (the concept that free radicals and reactive oxygen species act as biological messengers) [299]. Redox signalling induction factors such as macrophage colony-stimulating factor (M-CSF) are required for the differentiation of monocytes to macrophages. Macrophages ingest oxidized LDL, and slowly turn into large foam cells that eventually die and propagate the inflammatory process [300]. There is also smooth muscle proliferation and migration from the tunica media into the intima responding to cytokines secreted by damaged endothelial cells (Figure 1.10). This causes the formation of a fibrous capsule covering the fatty streak (atheroma).

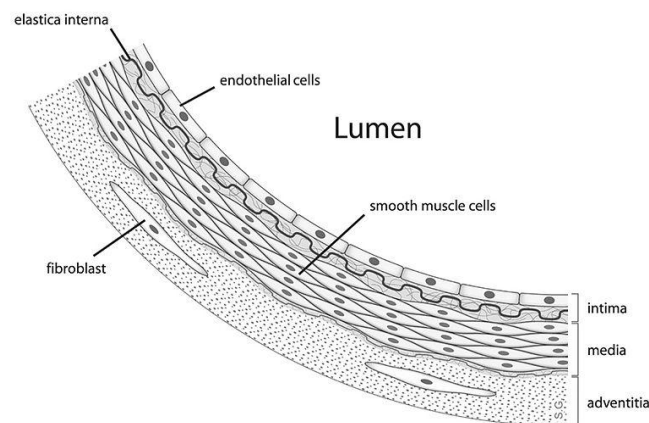


Figure 1.10 The anatomy of the arterial wall showing the layers of cells within the intimal and medial layers of the arterial lumen. Accessed 2011 (www.boundless.com).

1.11 Vascular calcification

Vascular calcification is associated with congestive heart failure, hypertension, myocardial ischemia, and coronary insufficiency [301]. The process of VC is varied, occurring in different parts of the vessel and causing damage in different ways. For example, calcification in the vessel wall occurs in two sites: the intima and the media. Intimal calcifications are a consequence of the inflammation and calcification of atherosclerotic

plaques. Beneath the endothelium there is a fibrous cap covering the atheromatous core of the plaque and the presence of these plaques is generally associated with atherosclerotic burden, endothelial dysfunction and rupture leading to the formation of the thrombus [302]. Medial calcifications on the other hand are frequently found in the elastic interna of the aorta and coronary arteries, and appear to be independent of atherosclerosis, but may coexist with it. This type of calcification is also known as “Mönckeberg sclerosis”, after Johann Georg Mönckeberg who first described it in 1903 [303]. Medial calcifications occur frequently in patients with CKD and DM, but also occur frequently within the ageing population [302-304].

1.11.1 Pathophysiology of vascular calcification

The mechanism by which the process of VC occurs is complex. It does not consist of a simple precipitation of calcium and phosphate but is instead an active and modifiable process in which the vascular smooth cells (VSMC's) undergo cell death (apoptosis) and vesicle formation changes the phenotype of smooth vascular cells into osteoblast-like cells. This induces matrix formation and also attracts cells that are involved in the mineralisation process [302]. Vascular calcification is often triggered by active processes involving inflammatory cytokines such as TNF- α , C-reactive protein (CRP) and proteins CD40 - CD154 [305, 306] and may also be induced by disorders with calcium and phosphate (Ca/P) metabolism [305, 306], initiating VSMC damage and subsequent phenotypic switch with activation of bone forming programs [303]. Four types of calcification can be distinguished on the basis of location, association with plaque, and mode of formation [307]: Dystrophic or passive calcification - a minor form of widespread non-specific organ and soft tissue calcification as a result of abnormal Ca/P product, and three morphologic types of actively regulated calcification (in the absence of raised Ca/P levels), i) calcification of cardiac valves, ii) calcification in arterial intimal layers in association with macrophages, lipids, and VSMC as in classical atherosclerosis, iii) calcification in arterial medial layers as a result of elastin fibre mineralisation, VSMC degeneration, and upregulation of osteogenic programs as in CKD or diabetes. The bone regulating proteins including OPN, OPG, MGP, Fetuin-A and receptor activator of NF- κ B [308-310] and RANKL have been identified as playing major roles in the calcification developmental process [307].

1.12 Techniques used for measuring vascular calcification

A number of non-invasive imaging techniques are now available to detect and quantify VC. Plain radiographs of the abdomen and extremities are used to identify macroscopic

calcifications of the aorta and peripheral arteries [311-313]. Techniques such as echocardiography and 2-dimensional ultrasound are reliable and widely utilised methods for the assessment of valvular calcification [314, 315], and calcification of the carotid arteries, femoral arteries and aorta [316]. Computed tomography is considered to be the 'gold-standard' for qualitative and quantitative assessment of coronary and aortic calcification (AC), and is a useful method of monitoring the progression and effects of therapeutic strategies aimed at modifying the progression of calcification [317, 318]. Although these imaging techniques are able to detect and quantify VC it is important to highlight a substantial limitation - that none of the techniques described are able to accurately differentiate between medial (arteriosclerotic) and intimal (atherosclerotic) calcification. For example, medial calcification is associated with arteriosclerosis which is characterised by the thickening, hardening and loss of elasticity of the arterial wall, whereas intimal calcification is associated with atherosclerosis and is characterised by the development of fatty plaques that subsequently obstruct the arterial lumen; It is likely that the quantification of VC using the imaging methods described will result with the measurement of either intimal or medial calcification or in some instances both types where present.

1.12.1 Ultrasound

Ultrasound (US) based imaging methodologies have been widely adopted to study VC in dialysis patients to assess superficial vessels, such as the femoral and carotid arteries [319]. Ultrasound involves the transmission of high frequency sound waves (2 to 10 MHz) through an anatomic site of interest followed by conversion of echoes into electrical impulses, producing 2-dimensional images [320]. Ultrasound studies rely on the availability of the tool, the inexpensive nature of the measurements and the ease of identification of superficial vessels such as the carotid and femoral arteries. Ultrasound-based methods, however, only provide qualitative and semi-quantitative assessment of VC [321, 322]. The distinction between intimal and medial calcification is difficult and results are, for the most part, based on subjective interpretation [323]. However, the results generated with this methodology appear to be a reliable means for VC screening and outcome prediction. The advantages of US are that it is a safe method with no radiation exposure, and is relatively low cost, moreover, it permits assessment of calcification of superficial vessels such as carotid and femoral arteries [324]. It is however operator dependent and only a qualitative method [324].

1.12.2 Aortic Pulse Wave Velocity

Arterial walls stiffen with age and the primary structural change with ageing is medial degeneration, which leads to progressive stiffening of the large elastic arteries [325]. The media of large arteries is composed of VSMC's and elastic and collagen fibres, comprising functional musculoelastic sheets [326]. Arterial pulsation has a direct effect on collagen and elastin in the arterial wall, disrupting muscular attachments and causing elastin fibres to fatigue and fracture [325]. Another major change in the arterial wall is that of calcium deposition; the calcium content of the arterial wall increases with age, particularly after the fifth decade, which also contributes to arterial stiffness [327]. A well-validated method for the assessment of arterial stiffness is pulse wave velocity (PWV) [328, 329]. Increased PWV is related in part to the extent of calcium within the artery and is a simple, non-invasive, radiation free method for estimating VC. Pulse wave velocity can be measured in any arterial segment between two regions with carotid-femoral PWV is the most widely used method for assessing central arterial or aortic stiffness [330] which is shown to be an independent predictor of CV mortality and morbidity in the elderly [331, 332], as well as in the general population [333, 334]. The assessment of aortic PWV involves the measurement of two quantities: the transit time of the arterial pulse along an arterial segment and the distance between both recording sites (Figure 1.11).

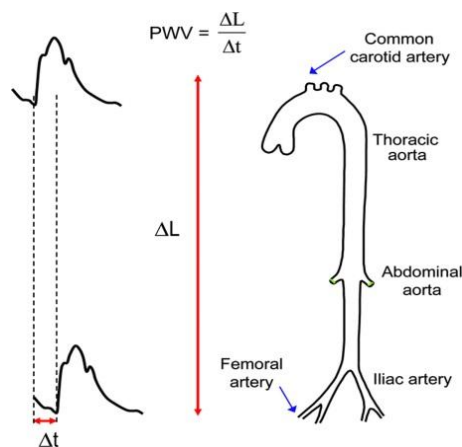


Figure 1.11 Carotid-femoral pulse wave velocity (PWV). Image shows the transit time of the arterial pulse along an arterial segment (the aorta) and the distance between the carotid and femoral pulse recording sites. Accessed 2012 (www.neurology.org).

Pulse wave velocity is calculated as the distance travelled by the pulse wave divided by the time taken to travel the distance [335] and increased arterial stiffness results in an increase in speed of the pulse wave within the artery. The advantages of PWV are that it

is safe to use and offers good reproducible results with a coefficient of variation (CV %) between 8-12% [335-338].

1.12.3 Radiographs

Plain radiographs of the chest, abdomen and extremities are routinely collected in clinical practice and represent a valuable and inexpensive tool for detection of VC in both the general population [339] and in patients with chronic kidney disease (CKD) [340]. Lateral lumbar radiographs can also accurately detect AC and careful examination may even distinguish between intimal and medial calcification [313]. Medial calcification is usually characterised on plain radiographs as linear lesions visible along the course of an artery (Figure 1.12), whereas intimal calcification is more characteristically identified by patchy and irregular radio-opaque lesion [341].



Figure 1.12 Plain radiograph showing medial calcification of the femoral artery (arrows in black).

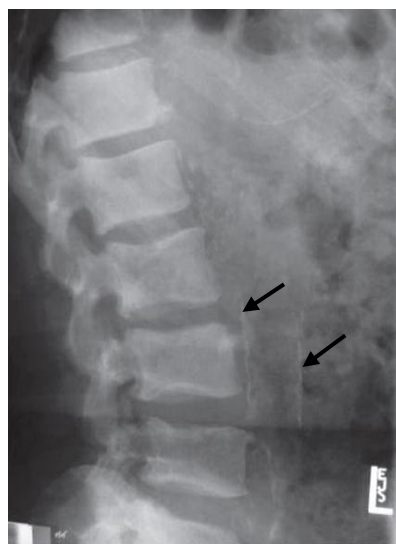


Figure 1.13 Lateral lumbar radiograph showing linear calcifications (arrows in black) along the wall of the abdominal aorta in front of lumbar vertebrae 1–4.

Although plain radiographs are mostly qualitative, several semi-quantitative methods have been developed to assess VC. The best known of these methods is that by Kauppila et al [342], whereby AC visualised on a lateral radiograph of the lumbar spine is given a score of 0–24 based on the number and extent of calcified deposits in the aortic segments extending in front of lumbar vertebrae 1–4 (Figure 1.13). The advantages of plain radiographs are that they are low cost, and it may be possible to determine intimal from medial calcification based on characterisation of the plaques by the reader [341]. The

disadvantage is that they only provide a qualitative or semi-quantitative assessment of VC, and the scoring method used is reader dependent [341].

1.12.4 Magnetic resonance Imaging

Magnetic resonance imaging (MRI) is a radiation free, non-invasive method of measuring VC and is capable of distinguishing various components of atherosclerotic plaques such as fibrous tissue, lipids, calcification, and thrombus [343-345]. MRI employs the use of nuclear magnetic resonance (NMR) to image nuclei of atoms inside the body. NMR is a physical phenomenon in which nuclei in a magnetic field absorb and re-emit electromagnetic radiation. Magnetic resonance imaging can create more detailed images of the human body than possible with X-rays. The quantitation of plaque dimensions and components is calculated using a 3-dimensional image reconstruction [346].

1.12.5 Computed Tomography

Computed tomography is an imaging technique that utilises computer-processed X-rays to produce tomographic images or 'slices' of specific areas of the body. Digital geometry processing is used to generate a three-dimensional image of the inside of an object from a large series of two-dimensional X-ray images taken around a single axis of rotation [347]. Computed tomography produces a volume of data that can be manipulated, through a process known as windowing, in order to demonstrate various bodily structures based on their ability to attenuate the X-ray beam. This information is interpreted as Hounsfield Units (HU). Although historically the images generated were in the axial or transverse plane, perpendicular to the long axis of the body, modern scanners allow this volume of data to be reformatted in various planes or as volumetric (3D) representations of structures.

Electron beam computed tomography (EBCT) and multi detector computed tomography (MDCT) are well-validated, non-invasive imaging methods which do not require the administration of radio-opaque contrast dyes [348]. The methods are considered to be the gold-standard for assessing the extent of VC and its progression in the coronary arteries, the aorta and the cardiac valves [349, 350]. Both CT technologies are considered equivalent in accuracy and reproducibility even though they operate on different imaging platforms [351-353]. Electron beam computed tomography employs a rotating fan of X-rays produced by the impact of a beam of electrons against a tungsten ring. Image acquisition is rapid, limiting patient exposure to radiation [348]. Multi detector computed tomography employs a paired X-ray source-detector unit revolving in a spiral motion

around the patient who lies on a movable bed that advances through the beam of X-rays [348]. Although MDCT is slower than EBCT and provides a higher radiation dose, it has a higher spatial resolution (better image quality) than EBCT [354]. Despite the advantages of CT imaging techniques for the quantification of calcification, there are also substantial limitations. The method is unable to distinguish intimal from medial calcification and CT methods are expensive and provide increased exposure to ionized radiation. The lack of accessibility of scanners can present a major obstacle to its routine application and more importantly the method requires a clinician or highly trained and experienced reader to interpret the results.

1.13 Cardiovascular disease and osteoporosis.

It is now widely accepted that both OP and CVD are common conditions and are a major cause of morbidity and mortality within the ageing population. Traditionally they have been viewed as two separate conditions that increase with age and are therefore frequently seen in the same individual [355-363]. The suggestion of a relationship between OP and CVD was reported in 1990 [364], and furthermore a study of 115 postmenopausal women who had CT and DXA measurements showed reduced BMD in those with AC [365]. Recent evidence suggests that there are direct associations between BMD and VC, atherosclerotic burden and CV related events and mortality [356-363, 366, 367], independent of age and traditional CV risk factors.

1.13.1 Cardiovascular disease and bone loss

Subclinical or preclinical atherosclerosis refers to the early stage of the process of atherosclerosis where within the vascular walls pathological changes are taking place. Increasing evidence suggests that subclinical atherosclerosis confers an increased risk of CVD [368]. Studies have evaluated the association between subclinical atherosclerosis and OP and both men and women with progressive AC have significantly higher bone loss in the lumbar spine compared with subjects without AC progression [369]. These findings correlate with other studies where AC progression is associated with higher rates of bone loss in the proximal femur and metacarpal bones [370, 371]. Furthermore, several prospective studies showed that subjects with calcifications in the aorta, coronary arteries, carotid or femoral arteries have significantly lower BMD compared with subjects without VC [356, 360, 372-377]. Conversely, a few studies failed to find an association [378-381]. Intima-media thickness (IMT) is used to detect the presence of atherosclerosis and may be used to track the regression, arrest or progression of atherosclerosis [382] and studies examining the relationship between IMT and BMD reported an association between

increased IMT and low BMD [383-390]. Altogether, study data strongly suggest that subjects with subclinical atherosclerosis and early CV disease are at increased risk of bone loss.

1.13.2 Cardiovascular disease, bone mineral density and fracture risk

Several studies have examined the relationship between CVD and fracture risk [369, 370, 391, 392]. An increased risk of incident fracture was observed in five studies [370, 391-394]. The largest of the studies included more than 30,000 twins with a follow-up duration of 20 years [393]; an increased hip fracture risk was reported after all diagnoses of CVD in both men and women. Furthermore, the study showed that CHD and cerebral vascular disease was associated with an increased risk of fracture [393]. Studies have also evaluated the association between peripheral artery disease (PAD) and fracture risk, and reported PAD to be associated with an increased risk of non-vertebral fractures [391] and hip fractures [393]. Another large study of over 2000 healthy postmenopausal women by Bagger et al showed that AC was a strong predictor for fragility fractures; AC predicted a 2.3-fold increased risk for hip fracture [370]. It has also been reported that not only women, but also men with advanced AC have a two- to three-fold increased fracture risk [394]. In 2005 Tankó reported data on fracture risk from a large study cohort of 2500 postmenopausal women and concluded that not only did women with OP at the spine have a four-fold increase in the risk of a CV event occurring compared to those with osteopenia, but also it demonstrated that women with at least one vertebral fracture had a three-fold increase in the risk of a CV event as the severity of OP increased [361]. A study by Schulz et al examined the link between AC and the risk of osteoporotic fractures and concluded that AC is a strong predictor for low BMD and fragility fractures [359].

1.13.3 Low bone mineral density and cardiovascular morbidity

Numerous studies have investigated the association between BMD and CV morbidity [361, 395-397]. A cross-sectional analysis from the Health, Ageing, and Body Composition (ABC) Study showed volumetric BMD (vBMD) measurements of the spine were significantly and inversely associated with prevalent CVD in men and women [398]. Other studies have reported significant associations between OP and CVD in women [399]. For example, in a retrospective analysis of a predominantly female population referred for angiography and BMD assessment, OP was reported to be associated with angiographically-determined CAD [397]. A report from the 30 year follow-up of the Framingham study found that metacarpal cortical area (MCA) predicts CHD in women free

from CVD at baseline, with a significant trend of decreasing CHD risk with an increase in MCA [392].

1.13.4 Low bone mineral density and cardiovascular mortality

Low BMD and bone loss appear to be risk factors for CV mortality in both men and women and several prospective studies have reported low BMD to be inversely related with CV mortality [358, 366, 395, 400-402]. Postmenopausal women with low BMD had a 1.2- to 2.3-fold increased risk of dying from CV events, independent of traditional CV risk factors [366, 384, 403]. The Study of Osteoporotic Fractures (SOF) showed that an increase in loss of BMD at the hip was associated with a 1.3-fold increase in CHD mortality among Caucasian women of 65 years and older [404]. Furthermore, in a study by Kado et al in 2000 calcaneal bone loss was related to increased risk of death due to atherosclerosis and CHD [358]. A prospective study by Triveldi et al in 2006 reported low BMD at the hip to be a significant predictor of CV mortality in a cohort of British men aged 65-76 years [402] and this association was independent of traditional CV risk factors [402].

1.14 Common pathogenesis linking osteoporosis and cardiovascular disease

Evidence from experimental research studies indicates a shared pathogenesis between OP and CVD [405]. Many of the factors that influence bone metabolism are involved in the development of vascular disease, namely atherosclerosis and VC, and several bone-related proteins are involved in the calcification process resulting in mineral deposition [406]. Vascular calcification is considered to be an active process, and like osteogenesis, involves a complex interaction between various cells that produce matrix vesicles and mineralisation [355]. Vascular smooth muscle cells are able to re-differentiate into osteoblast-like cells, and a subpopulation of cell known as calcifying vascular cells (CVCs), were shown to form nodules and mineralisation spontaneously [407]. In vitro, these osteoblastic cells produce hydroxyapatite, a mineral important in bone formation [408]. Paracrine regulators of bone metabolism such as MGP, OCN, BMP, OPN, ON, OPG, receptor-activated nuclear factor-kappa B ligand, and inflammatory cytokines are also present in atherosclerotic arteries [409]. In addition, osteoclast-like cells have been demonstrated in calcified arteries [410]. Thus, the vascular microenvironment possesses mechanisms to maintain the pathological development of mineralised deposits similar to that of bone tissues [355].

Oestrogen deficiency is a major risk factor for OP and CVD. After the menopause, oestrogen levels decrease dramatically resulting in osteoclast formation and an increase in bone turnover with subsequent rapid bone loss. In addition, the decline in production of oestrogen causes the secretion of pro-inflammatory cytokines such as IL-6, IL-1, and TNF- α [411, 412].

Bone morphogenetic proteins are members of the transforming growth factor (TGF- β) super family, and are important factors in the regulation of osteoblast differentiation [405]. Bone morphogenetic protein acts through up-regulation of transcription factors important in bone metabolism [405]. In animal models BMPs have been shown to inhibit the expression of collagenase-3 by osteoblasts, resulting in reduced collagen degradation and maintenance of bone mass [413]. Bone morphogenetic protein also appears to be an important mediator in VC as increased expression of BMP2 and BMP4 has been found in atherosclerotic lesions in endothelial cells, foam cells and VSMCs [408, 409]. In-vitro studies have shown factors known to induce CV disease, such as oxidative stress, oxidized low-density lipoprotein (ox-LDL) and TNF- α , are able to upregulate BMP expression in endothelial cells [414, 415].

Matrix Gla protein is a calcium-binding protein that requires vitamin K to fully function, and is found to be expressed in areas with VC [355]. Matrix Gla protein may be an important calcification inhibitor as animal studies have shown that MGP knockout mice develop extensive CAC [416]. Recently the mechanism by which MGP inhibits calcification has become clear; in vitro, MGP inhibits calcification by binding to BMP2, blocking the induction of osteoblasts [417]. However, the inhibitory effect of MGP on BMP-2 depends on the extent of MGP γ -carboxylation rather than the amount of MGP and It is likely that the lack of function from insufficient γ -carboxylation increases the risk of VC [418].

Osteopontin is a multifunctional glycoprotein that is expressed and secreted by various cell types including macrophages, endothelial cells, smooth muscle cells and epithelial cells. Osteopontin is also highly expressed in bone by pre-osteoblasts and osteoclasts [409] and accumulates in the extracellular matrix of bone tissue where it binds to hydroxyapatite and calcium [405]. Osteopontin is also found to be highly expressed in atherosclerotic arteries especially in association with macrophages and foam cells, suggesting that OPN plays an important role in the development and progression of atherosclerosis [409, 419]. It is not entirely clear whether OPN promotes or inhibits calcification in the arterial wall [420], however an experimental animal study by Speer et al suggests that OPN is an important negative regulator of calcification [421]. It has been suggested that OPN directly inhibits calcification as a consequence of tightly binding to

hydroxyapatite in VSMCs in much the same way as it binds to hydroxyapatite and calcium in bone [421]. In animal studies conducted under calcification-inducing culture conditions, VSMCs isolated from OPN-knockout mice demonstrated significantly higher calcification, suggesting that OPN in VSMCs is an important inhibitor of calcification [422]. In contrast, it is suggested that elevated OPN serum levels are associated with VC [423] and experimental studies have shown that vitamin D₃ increases OPN and calcification in bovine VSMCs [424].

Alkaline phosphatase is an enzyme found on the surface of osteoblasts and is used as a marker for bone turnover. The Hydrolysis of pyrophosphate, which is an inhibitor of hydroxyapatite formation, is needed to assist normal mineralisation [425]. Atherosclerotic calcification may be regulated by the induction of ALP in VSMCs in response to inflammatory mediators produced by macrophages and T cells present in plaque lesions [426]. In vitro studies on VSMCs showed that the ALP expression is increased in response to inflammatory markers, LDL and oxidative stress, and the increased expression was associated with increased mineralisation within the vascular wall [427, 428]. Upregulation of ALP expression has been demonstrated in the aorta of uremic rats made by feeding adenine [429]. However, factors responsible for the upregulation of ALP in uraemia remain to be clarified [430].

One important biochemical factor involved in bone resorption is OPG. Although not used as a bone marker in clinical practice, OPG may play an important role in the possible link between osteoporosis and VC. Osteoprotegerin production by osteoblasts is regulated by a number of factors, including BMP-2, inflammation, oestrogen, vitamin D and oxidative stress [427]. Osteoprotegerin is expressed in various tissues, including the skeleton and vascular wall and serves as a soluble decoy for RANKL [431]. Receptor activator of nuclear factor kappa-B ligand, its signalling receptor RANK, and its natural decoy receptor OPG are members of the tumour necrosis factor (TNF) and TNF receptor superfamily and are best known for their essential role in controlling osteoclastogenesis [432] (Figure 1.14).

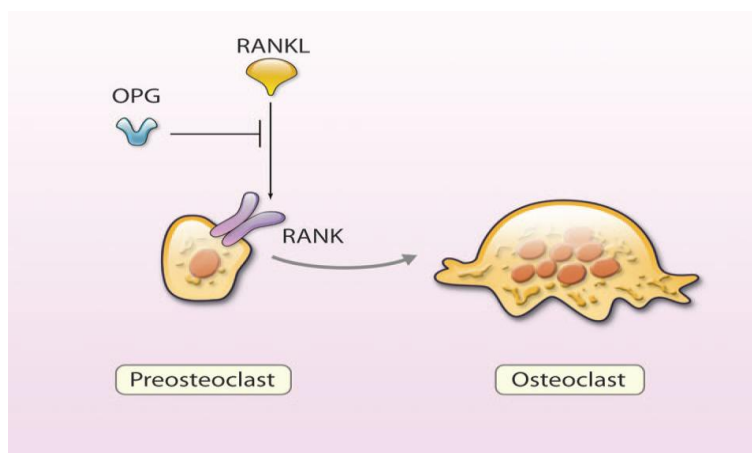


Figure 1.14 showing RANKL/RANK/OPG interactions. When RANKL from osteoblasts binds to its receptor, RANK, on the surface of preosteoclastic cells, it induces osteoclastic differentiation. OPG, also from osteoblasts, acts as a soluble decoy receptor for RANKL and, thus, blocks osteoclastic differentiation. Accessed 2012 (circres.ahajournals.org).

OPG knock-out mice show early-onset OP and increased VC [410]. In-vitro studies have shown that OPG may be important for endothelial cell survival [433] and may inhibit active calcification [434]. Experimental studies have shown that OPG might protect against VC, however, OPG levels appear to be elevated in patients with CV disease. Several studies have reported a correlation with high serum OPG levels and more severe CV disease [383, 404, 435-438]. While OPG appears to play a role in the pathogenesis of atherosclerosis, the exact mechanism remains to be explained [439].

Another important mechanism linking CV disease and OP is Wnt signalling [440]. Wnt signalling is crucial for osteoblast differentiation and bone formation and the Wnt- β -catenin pathway plays a vital role in bone metabolism [440]. Recent studies have implicated the Wnt signalling pathway in the pathogenesis of VC [440-445]. Animal models have demonstrated the important role of Wnt signalling in bone formation through lipoprotein receptor-related protein 5 (LRP5), lipoprotein receptor-related protein 6 (LRP6) and β -catenin [446]. Tumour necrosis factor- α is a cytokine involved in systemic inflammation, and like oxidative stress and vitamin D, it is shown to promote VC through the Wnt signalling pathway [447-449]. Wnt-signalling inhibitors, Dickkopf-1 (Dkk1) and sclerostin (SCLE) have important roles in bone turnover. Dkkopf-1 modulates bone formation via the Wnt pathway preventing an excessive formation of bone [450], and SCLE has a role in the reduction of bone formation via the Wnt pathway [451]. Furthermore, both Dkk1 and SCLE have been shown to be up-regulated in areas of VC [443, 452, 453]. This important research data helps to support the hypothesis that Wnt

signalling is an interesting new molecular mechanism that influences both bone and vascular metabolism [440].

1.15 The relationship between lipids, BMD and vascular calcification

Evidence suggests that CVD and OP share an etiologic factor – that hyperlipidemia contributes not only to atherosclerotic plaque formation, but also plays a role in OP, following a similar biologic mechanism involving lipid oxidation [454, 455]. There have been numerous studies reporting the relationship between lipids and low BMD [454-461]. The artery wall contains cells capable of differentiation into osteoblasts following the same stages of differentiation that occur in bone-derived osteoblasts and ultimately producing bone mineral [454]. It is known that oxidative stress alone promotes vascular-cell calcification [428] and may account for the pro-calcific effects of oxidized lipids [462]. Oxidative stress markers are also significantly associated with BMD [463]. The oxidant stressor, H_2O_2 , promotes osteochondrocytic differentiation of VSMCs by upregulating *runx2*, a key transcription factor associated with osteoblast differentiation [464]. In bone, high lipid levels inhibit osteoblastic activity resulting in reduced bone formation [454, 455, 458]. Experimental research has shown that products of lipid oxidation, such as minimally-modified LDL and oxidized phospholipids, induce osteogenic and apoptosis-mediated calcification of vascular cells [454, 465, 466].

Growing evidence suggests a negative effect of an atherogenic lipid profile on bone formation. In a cohort of postmenopausal women, plasma levels of LDL-cholesterol and HDL-cholesterol were negatively and positively related to BMD respectively [467]. Furthermore, studies have shown that patients with low BMD not only have high lipid levels but also a more severe coronary atherosclerosis independent of age, and as a consequence, a greater risk of a CV event including stroke [468]. Animal studies have demonstrated that lipids have been shown to accumulate in bones of mice [455] and mice fed an atherogenic high-fat diet were found to have reduced bone formation [469]. Once outside the plasma, these lipid products are subjected to oxidative modification, thus becoming biologically active molecules capable of affecting a variety of cellular processes that ultimately result in atherogenesis and bone loss [469]. In view of the accumulating evidence for a cross-link between the role of lipids in bone metabolism and VC what is known from data from clinical studies is that patients with lower bone density and OP also have higher lipid levels, more severe coronary atherosclerosis, and have a greater risk of stroke death [356, 362, 470, 471].

1.16 Aortic calcification, arterial stiffness and BMD

Calcification of the AA has been documented since the 1930's. A study by Kiel et al reported that the presence of calcified lesions in the AA has been shown to predict CV events [472]. Wilson et al reported the impact of AAC on the prediction of CVD and concluded that AAC deposits, detected by lateral lumbar radiographs, are a marker of subclinical atherosclerotic disease and an independent predictor of subsequent CV morbidity and mortality [339]. A recent large meta-analysis by Gonçalves et al in 2012 investigated the incremental value of AAC in predicting long term CV outcome [473] and concluded that the presence of AAC is a relevant and independent predictor of future CV events [473]. Arterial stiffening and has long been associated with the ageing and elderly and is known to be accelerated by CV risk factors including hypertension, diabetes, and hypercholesterolemia and more importantly end stage renal failure in CKD [329, 357, 474-479]. Furthermore, increased arterial stiffness is associated with OP and systemic inflammation [329, 357, 383, 478, 480]. Increasing arterial stiffness leads to an increase in systolic blood pressure (BP) and pulse pressure and a decrease of diastolic BP, resulting in increased left ventricular (LV) after-load and altered coronary perfusion [481] and the principal outcomes of these changes are left ventricular hypertrophy (LVH), coronary ischaemia, and an increase in fatigue in the structures of the arterial wall [481-483].

Pulse wave velocity is an important measure of arterial stiffness. Pulse wave velocity measures the velocity of travel of the pulse along a length of artery, usually between the carotid and femoral arteries to provide a measure of aortic stiffness - VC causes an increase in the rigidity of the vascular wall resulting in a deceleration of the progression of a pulse wave along the vascular wall. Numerous studies have shown that PWV is an important predictor of risk of CV events [455, 475, 476] so much so that it is a highly recommended screening tool for predicting CV risk particularly in ESRD [484-488]. Calcification is a common complication of CKD and ESRD [316, 489, 490]. In the general population and patients with ESRD, the extent of VC is associated with aortic stiffening and has shown to be predictive of subsequent CVD and mortality beyond established conventional risk factors [318, 321, 339, 491]. Disturbances in calcium (Ca) and phosphate (PO₄) metabolism are associated with uremic bone disease in CKD and ESRD patients, and the results of several studies indicated that Ca overload is associated with the presence and progression of AC [318, 492]. The evidence strongly suggests complex interplay among bone activity, Ca load, and AC development, but whether the bone activity is involved in the Ca load on AC is unknown [317, 484].

1.17 Bisphosphonates and aortic calcification: recent animal and clinical studies.

Bisphosphonates have been an established treatment for bone loss, OP and hypercalcaemia for many years. Based on experimental data, BPs appear to be promising agents for reducing the progression of human VC. The concept of the common underlying pathogenesis of OP and VC reinforces the idea that pharmacological agents that inhibit bone loss could potentially provide additional benefits by slowing down the progression of atherosclerosis [493]. In this context, BPs may be a possible therapeutic option for VC resulting from atherosclerotic disease [494, 495].

Bisphosphonates have been shown to be effective in treating and preventing OP by acting on the body's main source of hydroxyapatite, the skeleton [496]. Furthermore, BPs are also effective treatments for diseases associated with osteoclast-mediated bone resorption such as Paget's disease, multiple myeloma, and osteolytic tumour metastasis [493]. Following the deposition of new bone matrix, BPs are ingested by osteoclasts where they induce apoptosis, leading to decreased bone resorption [497]. Pharmacologically, BPs have a short half-life and are not metabolised in the body. Up to 60% of the absorbed amount rapidly enters bone or becomes excreted through the kidneys. Small quantities of BP may be incorporated into the spleen and liver [497] and also into arterial walls [498]; BPs have been located in calcified matrix outside the bone and have been identified in calcified atherosclerotic plaques in both animals and humans [497-501]. The accumulation of BP in atherosclerotic arteries is likely due to their affinity for calcium and hydroxyapatite which has been identified in calcified atheromatous lesions [493]. However, BPs have been found in high concentrations within the aorta without atherosclerotic lesions in mice and in healthy human mammary arteries [498] suggesting an alternative mechanism of action.

Great interest developed as a result of experimental findings that BPs inhibited extra osseous and soft tissue calcification in animal models [502, 503]. It has been reported that some BPs not only inhibit AC but also reduce lipid accumulation and fibrosis in atherosclerotic lesions in animal models [504], suggesting the potential for atherosclerotic disease regression. In addition, studies suggest that BPs may have an anti-atherosclerotic action which is independent of the lowering of cholesterol or calcium levels in circulation. From four of the main animal studies [503, 505-507] it has become clear that BPs have a protective role on the arterial wall, not only reducing calcifications but also the amount of lipid containing plaques regardless of diet. The mechanisms for this are unknown, however, what is known is that BPs accumulate in vascular tissue [508] and macrophages that are involved in the development of plaque lesions on the arterial wall are able to

internalise BPs that may induce apoptosis. It is also recognised that nitrogen-containing BPs inhibit an enzyme involved in the cholesterol biosynthesis pathway [504].

Although limited in number, clinical studies have investigated the effects of BPs on atherosclerosis and VC in humans, reporting favourable or neutral results [509-517]. Although BP therapy has shown to be effective on VC in animal models, there is still debate over its efficacy on VC in humans, with results differing between the different types of BP [516]. In studies on patients with ESRD undergoing dialysis, etidronate has been found to limit the progression of VC [510]. However alendronate and ibandronate have shown mixed results at limiting the progression of VC, with animal studies suggesting that they slow progression, while randomised controlled trials show no effect in humans [515].

Carotid artery intima-media thickening (CIMT) has been evaluated in 4 studies using alendronate, etidronate and risedronate [509, 518-520]. Two studies [509, 510] reported a significant reduction in CIMT following the use of etidronate and alendronate after 1 year. However, in contrast, Delibasi et al reported no significant change in CIMT after 1 year in postmenopausal women on 70mg of weekly alendronate [520]. Kanazawa et al reported a halt in the progression of CAC, identified by plaque scores, with the use of risedronate [509]. Coronary artery calcification measured in haemodialysis patients taking etidronate was reported to be reduced following 1-years treatment [510]. In contrast, a study by Ariyoshi et al, reported no significant difference in CAC scores in haemodialysis patients receiving etidronate [511]. Three studies have reported the positive effects of etidronate on AC measured by calcification scores [511-513] following 1-years treatment. A large study by Tanko et al, studied the effects of IV ibandronate in women with postmenopausal OP over a 3-year period. The study reported that ibandronate did not have any influence on the progression of AC over the 3-year follow up [514]. It is clear from the research that BPs have an inhibitory effect on the atherosclerotic process, however, there is still a need for large placebo-controlled studies to confirm this. Many of the studies with the exception of the study by Tanko [514], have been small in sample size with a short duration follow up. In addition, only a few studies have been placebo-controlled. Furthermore, most of the study subjects were elderly with independent CV risk factors such as DM and CKD [515].

Many of the underlying mechanisms for the association between VC and bone metabolism are still remain unclear. Previous clinical studies investigating the effects of BPs on VC have been limited to measurements of CIMT and calcification in the coronary arteries. However, laboratory studies have identified a cell population called endothelial progenitor cells (EPCs) that can be isolated from circulating mononuclear cells [516, 517,

521], bone marrow [522], and foetal cord blood [523]. EPCs reside in bone marrow and may be a potential candidate for the link between bone metabolism and the vascular system due to their mobilisation and response to vascular injury and their contribution to vascular repair [524, 525], and more notably, their role in VC. The concept of osteogenic vs non-osteogenic EPCs may explain previous conflicting results in experimental studies in which treatment with EPCs or bone marrow mononuclear cells may accelerate vascular plaque formation instead of improving vascular function [526, 527]. Laboratory evidence suggests that EPCs express a number of endothelial-specific cell-surface markers and exhibit numerous endothelial properties [528, 529]. To further investigate the potential mechanisms by which BPs regulate VC, studies have examined the effects of BP on EPCs using flow cytometry to evaluate changes in co-expressed osteoblastic surface markers [530]. A recent study by Peris et al reported that EPCs co-expressing an osteogenic phenotype are significantly increased in patients with severe CAD or endothelial dysfunction, and in vitro these cells have been shown to mineralise [530]. Furthermore, BPs lower the expression of osteoblastic cell surface markers by circulating EPCs. Although more research is required in this area, results to date raise the likelihood that BPs may modulate the endothelial to mesenchymal transition, whereby endothelial cells differentiate into bone type cells [529, 531]. Studies have also investigated the effects of BPs on the proliferation, adhesion and migration of VSMCs [532]. It is well established that accelerated proliferation and migration of VSMCs from the media to the intimal are processes that are fundamental in the development of both intimal hyperplasia and atherosclerosis (Figure1.15) [533-536].

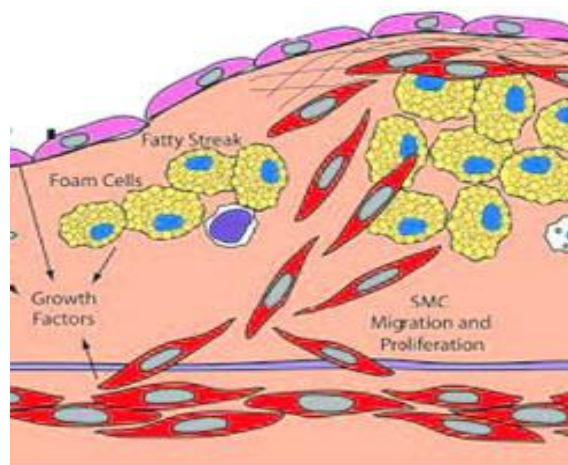


Figure 1.15 the migration and proliferation of vascular smooth muscle cells in an atherosclerotic lesion. Accessed 2012 (www.medicographia.com).

The inhibition of VSMC proliferation, adhesion and migration therefore represents a promising therapeutic strategy for the treatment of diseases such as atherosclerosis and intimal hyperplasia [532]. In an experimental study by Wu et al, zoledronate was found to have minor effects on VSMCs proliferation at relatively low concentrations. However, higher concentrations of zoledronate significantly reduced cell numbers [532], inferring the beneficial effects of therapeutic doses of BP on VSMCs. There is good evidence to suggest that BPs may inhibit the progression of VC, based on animal, clinical and experimental studies [510, 537-540]. What is also clear is that the action of the BP depends on the mechanism of development of VC, although exact mechanisms of BP on the different underlying mechanisms of VC still remain to be established.

Chapter 2

Methodology

2.1 Study overview

This chapter will outline the study design, objectives and hypothesis, and methodologies used. The research was conducted on site at Guy's Hospital, London in the Osteoporosis Unit and the Department of Nuclear Medicine. Ethical approval was obtained from St Thomas' Hospital Ethics committees for this study.

2.2 Study objectives

The primary study objective was to examine the relationship between BMD and AC in postmenopausal women.

Secondary objectives:

1. To evaluate two simple non-invasive techniques including pulse wave velocity (PWV), a measure of arterial stiffness and lateral DXA-VFA scans for quantifying AC by comparing to the 'gold-standard' of CT.
2. To investigate the associations between AC, BMD and regulators of bone remodelling including sclerostin (SCL), Dickkopf-1 (Dkk1), vitamin D and bone resorption and formation biochemical markers including Serum type 1 pro-collagen N-terminal (PINP) and serum collagen type 1 cross-linked C-telopeptide (CTX), in addition to serum lipids.
3. To determine if AC is significantly reduced in postmenopausal women on bisphosphonates by comparing with treatment naïve women.
4. To examine the effects of the bisphosphonate alendronate on AC in women with OP or severe osteopenia.

2.3 Study hypotheses

1. Lateral VFA scans of the lumbar spine and PWV, a measurement of aortic stiffness, offer simple non-invasive methods for identifying subjects at risk of AC and subsequent CVD, which could be implemented into the clinical management of patients with OP.

2. Low BMD is associated with a greater degree of AC in postmenopausal women.
3. Bisphosphonate users have less AC compared to non-bisphosphonate users.
4. Bisphosphonates decrease AC as well as increase BMD offering an effective treatment for both OP and VC.

In order to meet the research objectives as detailed in Section 2.2 the study was presented as five individual study chapters to test the study hypotheses as detailed in Section 2.3:

Chapter 3 - Intra- and inter-rater agreement of CT and lateral VFA images for the quantification of AC

This chapter evaluated lateral VFA imaging – primarily used for the assessment of vertebral deformity - to determine whether the method may offer a quick and reproducible method for quantifying AAC. Furthermore, this study evaluated the use of moderate dose, non-diagnostic, non-contrast CT as a reproducible alternative to higher dose diagnostic CT methods used in clinical practice to quantify VC, and also evaluated three calcium scoring methods to establish which would provide the most expedient and operator friendly method of scoring calcification using non-diagnostic CT.

Chapter 4 - Evaluation of lateral VFA images and pulse wave velocity for the assessment of AAC by comparison to the gold-standard of CT

The main aim of this chapter was to evaluate the accuracy, sensitivity and specificity of lateral VFA scans for the detection of AAC as measured using the 'gold-standard' method of CT. This study was performed in order to establish the reliability of lateral VFA imaging in clinical practice for quantifying AAC which could be performed concurrently with BMD scans, thus providing a quicker method of determining patients who may be at risk of future CV events. This chapter also explored the relationship between aortic stiffness measured using PWV ultrasound with AAC measured using lateral VFA imaging and VC measured using moderate dose, non-diagnostic, non-contrast CT to determine how well aortic stiffness correlates with AC in postmenopausal women.

Chapter 5 - Associations between BMD, AC and aortic stiffness in postmenopausal women

This study evaluated the associations between AC, arterial stiffness and BMD in a low risk population of otherwise healthy postmenopausal women, using non-invasive imaging methods including lateral VFA and moderate dose CT to quantify AC and VC and also PWV ultrasound to evaluate arterial stiffness. This study was performed in order to test

the hypothesis that low BMD is associated with a greater degree of AC in postmenopausal women.

Chapter 6 - Associations between regulators of bone remodelling, Dkk1 and sclerostin, BMD, VC and aortic stiffness in postmenopausal women

The purpose of this study was to investigate the relationships between regulators of bone remodelling (Dkk1 and sclerostin) with BMD, VC (measured using DXA and moderate dose CT) and aortic stiffness (measured using PWV ultrasound) to provide further evidence of their involvement in the pathogenesis of OP and VC – both of which are frequently observed in the ageing female population.

Chapter 7 - The relationships between BMD, VC and aortic stiffness between bisphosphonate users and treatment naive postmenopausal women: A cross-sectional and prospective study

This was a two part chapter using both cross-section and prospective data to compare VC and aortic stiffness in postmenopausal women taking BP therapy for the treatment of OP and in treatment naive women, and also to evaluate the effects of the BP alendronate on AC and aortic stiffness in postmenopausal women with low BMD over 12-months in a randomised, controlled trial. This study was conducted to test the hypothesis that the use of BP therapy may confer an inhibitory effect on VC which is of great clinical importance since no therapies are available that can reverse or stop the progress of VC.

2.4 Study population

A total of 462 Postmenopausal women with and without fracture were recruited on to the study following written informed consent. Subjects were aged between 50-81 (mean age 62) years with a wide range of BMD values including women classified as having normal BMD, osteopenia and OP according to the WHO criteria for OP [53]. Subjects were recruited from 3 sources:

1. Patients referred to the Osteoporosis Unit at Guy's Hospital by their GP for routine DXA screening for OP.
2. Patients attending the NHS Metabolic Bone Clinic at Guy's Hospital.
3. Volunteers from the general population who have contacted the Osteoporosis Unit at Guy's Hospital to volunteer to take part in OP research studies.

During the study consultation, each subject was required to complete a bone health and CV risk factor questionnaire.

2.4.1 Inclusion and exclusion criteria

Inclusion criteria

- Ambulatory postmenopausal* women.
- Aged 50 and over.
- With or without OP.
- With or without fracture.

Further inclusions for prospective study

- Lumbar spine, femoral neck and/or total hip BMD measurement more than 2 standard deviations below the young adult mean for healthy women (T-score \leq -2).
- Normal or clinically insignificant laboratory values

* Postmenopausal status for women with an intact uterus is defined as no vaginal bleeding for at least 12 months or having undergone surgical menopause by removal of the uterus and/or ovaries. Postmenopausal status for women who have had a hysterectomy and/or bilateral oophorectomy will be defined biochemically using serum estradiol and FSH levels.

Exclusion criteria

- Current or recent (within 1 year on enrolment) metabolic bone disorders other than postmenopausal OP including Paget's disease, renal osteodystrophy, osteomalacia or any other disease known to influence bone metabolism.
- Current uses of treatments known to effect bone turnover.
- Current use of lipid lowering agents.

Further exclusions for prospective study

- Previous or current use of oral or IV bisphosphonates
- Treatment with oestrogens, progestins or selective oestrogen receptor modulators (SERMS) for longer than 3 months in the past or any of these treatments within 3 months of study enrolment.
- Current treatment with corticosteroids, calcitonins, strontium ranelate, anticonvulsants, vitamin D > 50,000 IU/week, teriparatide, PTH1-84 or any other treatment known to influence bone metabolism.
- Abnormalities of the oesophagus and other factors which delay oesophageal emptying such as stricture or achalasia
- Hypersensitivity to alendronic acid or to any of the excipients
- Hypocalcaemia

2.5 Study design

The main aim of this project was to investigate the relationship between AC and BMD in postmenopausal women. The study was divided into three parts:

- 1. Preliminary imaging study** of 63 postmenopausal women to evaluate the novel application of imaging methods for the qualitative and quantitative assessment of AC and VC at other important vascular sites.
- 2. Cross-sectional study** of 444 postmenopausal women with a range of BMD levels, with or without fracture.
- 3. Prospective study** of 24 postmenopausal women to investigate the effects of alendronic acid on AC.

Table 2.1 Population numbers for VC imaging methods between study groups.

n=462	Total study	Cross-sectional study	Prospective study
<i>Imaging method</i>			
Lateral IVA	364	340	24
PWV	386	362	24
CT scan	137	113	24

2.5.1 Preliminary imaging study

The rationale for this part of the study was to evaluate the novel application of simple, non-invasive imaging techniques for quantifying AC. Two methods including PWV, an ultrasound method of assessing arterial stiffness, and lateral VFA scans, an imaging method traditionally used for the assessment of vertebral deformity, were compared to the 'gold-standard' of CT. Traditionally CT has been used for the qualitative and quantitative assessment of calcified deposits within the coronary arteries [541-546]. The aim was to investigate the reproducibility and the intra- and inter-rater agreement of moderate dose CT for quantifying VC in the AA and other important vascular sites. The study focused on the image acquisition and the test-retest reproducibility of the scan results and the intra and inter-reader agreement, which will be influenced in part, by patient positioning and patient size. In addition to the scan acquisition, the Agatston, modified Agatston and volume scoring methods were assessed in order to establish the best method for scoring AC. Eight postmenopausal subjects aged 65 and over were recruited to have repeat CT scans and 19 postmenopausal women aged 50 and over were recruited to have repeat

lateral VFA scans. This part of the study included women with a range of BMD levels including those with and without OP.

2.5.2 Cross sectional study

The rationale for this part of the study was to study the relationship between BMD and AC in 444 postmenopausal women; investigating associations between AC, BMD and regulators of bone remodelling that have been implicated in the pathogenesis of VC such as the Wnt signalling pathway. The number of participants was based on a formal power calculation. Assuming a correlation of 0.27 between BMD and AC, estimated using pilot data collected previously, then a group of 260 subjects was considered sufficient to demonstrate a statistically significant correlation with a Type-1 error of $p=0.05$ and 90% power. As the correlation between other important variable including biochemical markers would likely be lower, particularly in multivariate models, the study number was increased to 500 subjects to ensure adequate power to detect all correlations. Women aged 50 and over were recruited on to this study, with BMD values ranging between normal, osteopenic and osteoporotic according to the WHO criteria for OP [53]. Subjects that were taking treatments that are known to affect bone metabolism at the time of the assessments were excluded from the study, with the exception of BPs as one of the important aims of this part of the study was to compare AC and BMD in BP users with subjects that had never taken BPs.

Preliminary imaging study assessments

2 study visits:

Visit 1

- Bone density scans of the whole body, lumbar spine, hip and lateral VFA.
- Blood tests to measure lipid levels and bone markers that reflect the rate of bone turnover.
- PWV ultrasound scans to measure aortic stiffness.
- A full medical history.

Visit 2

Computed tomography scan of the chest and abdomen

Cross-sectional study assessments

1 study visit

- Bone density DXA scans of the whole body, spine, hip and lateral VFA.
- Blood tests to measure lipid levels and bone markers that reflect the rate of bone turnover.
- PWV ultrasound scans to measure aortic stiffness.
- A full medical history.

A sub-group of 100 subjects were selected to have CT scans to measure VC.

2.5.3 Prospective study

This was a single-centre, 2-year, randomised, open-label study to determine the effects of alendronate on AC. This research was clinically important since there are no therapies available that can reverse VC. Results will provide valuable information on whether BPs can be used to prevent or decrease AC in addition to improving BMD and reducing fracture risk. Since the effects of BPs on aortic stiffness (PWV) have not been previously investigated, a conservative value of 7.5% for the expected change in PWV had been estimated over the 2-year study period in response to treatment. Assuming a 7.5% standard deviation with a 7.5% change then a group of 21 subjects would be sufficient to detect statistically significant change with a Type-1 error of $p=0.05$ and 90% power.

Thirty subjects were recruited on to this part of the study, although 6 subjects failed at screening. Study subjects were randomised and divided into two groups (Figure 2.1).

Group 1 12 women to commence once weekly alendronate 70mg for 2-years and daily calcium and vitamin D supplementation providing 600mg/400IU of calcium and vitamin D.

Group 2 12 controls that were only given calcium and vitamin D supplements providing 600mg/400IU of calcium and vitamin D. The study subjects attended for a total of 7 visits over 2-years.

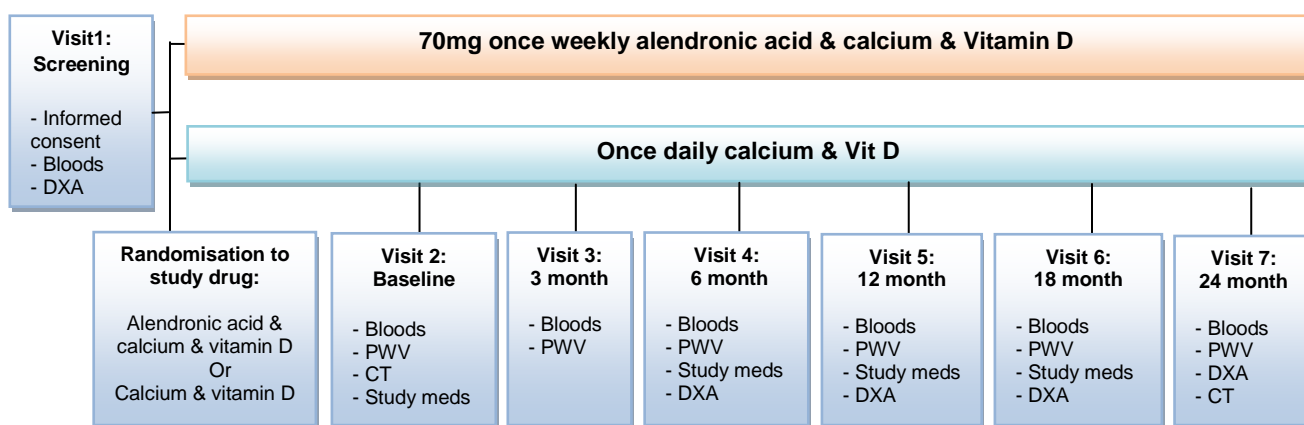


Figure 2.1 prospective study design outlining requirements at each study visit.

Randomisation onto the two study groups took place after the first visit once suitability was established. Suitability for the study was assessed using BMD results measured using DXA and haematological and biochemical parameters from blood testing, which included a full blood count (FBC), erythrocyte sedimentation rate (ESR), bone, lipid and thyroid profiles, thyroid stimulating hormone (TSH), parathyroid hormone (PTH), Vitamin D

and protein electrophoresis. Blood test results from screening were used to check for secondary causes of OP and any subjects that were found to have secondary causes were considered not suitable. Only subjects with a bone density T-score of -2.0 or lower at the total hip, femoral neck or lumbar spine were included. Treatment compliance was assessed at all follow-up visits following baseline based on the returned medication and calculated as:

Actual doses taken / expected doses x 100

All subjects took a maintenance dose of calcium and vitamin D supplements once a day providing 600mg calcium and 400 IU vitamin D for the duration of the study. Measurements of BMD, lateral DXA scans, PWV scans and serum markers of bone formation and resorption were performed at 6, 12, 18 and 24 months. Subjects also had an assessment of PWV and serum markers at 3-months. All study subjects consented to have CT scans at baseline and 24-months, providing 'gold-standard' information on changes in AC in response to alendronic acid therapy.

2.6 Trial Medication

The investigational product in this study was alendronic acid 70 mg tablets, which is a prescription-only medication for the treatment of postmenopausal OP. Alendronic acid 70 mg tablets contain the active ingredient alendronate sodium trihydrate and were provided in tablet form for oral administration. The strength of 70 mg once weekly is the standard dose for the treatment of postmenopausal women with OP.

2.6.1 Alendronic Acid

The brand of alendronic acid chosen had been decided by the hospital pharmacy (Guy's Hospital) according to local practice and guidelines at the start of the study. The brand used has marketing authorisation in the UK. The alendronic acid was stored and dispensed by the hospital pharmacy in the standard manufacturing packaging (i.e. open label) in cartons containing 4 tablets and each label included the study title, subject ID and dispensing date. Subjects commenced alendronic acid at the baseline visit (Visit 2). Dispensing took place at baseline, 6-months, 12-months and 18-months.

2.6.2 Calcium and vitamin D supplementation

All subjects, in both the alendronic acid and control groups, were provided with a stable dose of calcium and vitamin D supplementation from baseline until the end of the study. Calcium and vitamin D supplements were provided in tablet form for oral administration. These supplements provided a minimum of 600 mg/day elemental calcium and 400 IU/day vitamin D and were provided open-label. This minimum dose was that used for patients diagnosed with low bone density or OP. The brand used was not fixed and therefore decided by the hospital pharmacy according to local practice and guidelines at the start of the study. The calcium and vitamin D supplements were stored and dispensed by the hospital pharmacy in the standard manufacturing packaging (i.e. open label) in cartons containing 100 tablets. Each label included the study title, subject ID and dispensing date. Subjects commenced calcium and vitamin D supplements at the baseline visit (Visit 2). Calcium and vitamin D was also dispensed at 6-months, 12-months and 18-months.

2.7 Study methodology

2.8 Dual Energy X-ray Absorptiometry (DXA)

Dual energy X-ray absorptiometry is a widely accepted method for measuring BMD, assessing fracture risk, diagnosing OP, making clinical decisions about patient management and monitoring changes in BMD in response to therapy. This technique utilises two x-ray beams which have two different average x-ray energies that are projected through soft tissue and bone. The attenuation is measured at high and low energies. The area density is calculated at each point by two simultaneous equations, which then calculate bone mineral content (BMC), bone area and average BMD. In the present study all DXA scans were performed using standard local protocols for the measurement of bone density by a clinical scientist (SE) or experienced DXA radiographer. All study subjects had DXA scans of the lumbar spine (L1-L4) and hip to measure BMD and whole body DXA scans for the measurement of whole body BMD/BMC and body composition. In addition, each subject had a lateral VFA scan performed. The use of a C-arm meant that the lateral VFA scan could be performed supine. Scans were obtained using the Hologic Discovery QDR 4500 system densitometer (Hologic, Bedford MA) (Figure 2.2). The reproducibility for lumbar spine and proximal femur (hip) scans are approximately 1.0% (CV% at BMD = $1\text{g}/\text{cm}^2$) and precision for whole body scans is CV 0.75% [547].



Figure 2.2 the Hologic Discovery QDR 4500 densitometer at Guy's Hospital, Osteoporosis Unit used to perform DXA scans to measure BMD and body composition and lateral DXA-VFA scans to evaluate aortic calcification.

The DXA scanning system is comprised of two main elements; a table and a rotating C-arm (Figure 2.2). The table with a cushioned table pad provides a comfortable area in which the subject is positioned for scan acquisition and has clearly marked indicators to aid subject alignment (Figure 2.3). The rotating C-arm contains the x-ray source, x-ray detectors and laser, and the x-rays are generated underneath the table and projected upwards while detectors at the top of the C-arm convert the X-rays into electronic data which is then sent to the computer where it is converted to two-dimensional scan images. A red laser projects a visual cross-hair indicator onto the patient during patient positioning indicating the correct start point of the X-ray beam, aiding correct image acquisition.

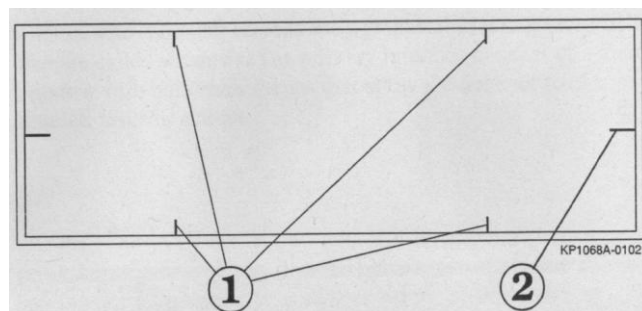


Figure 2.3 shows the scan table pad with alignment markings; 1: scan limit border, 2: centre lines used to centre the patient on the scan table. Modified from the Hologic QDR 4500 Discovery Operator Manual (www.hologic.com).

Prior to performing the study DXA scans, subjects were required to remove all metal including underwear with under wires & clasps in addition to any clothing that may have metal fastenings or zips. All subjects had height and weight measured to the nearest 0.1m

and 0.1kg respectively which was recorded on the Discovery QDR Apex image analysis programme on the DXA computer system. The level of radiation exposure was also recorded for each subject as required by the Ionising Radiations Medical Exposure (IRMER) Regulations. Effective dose is the preferred method of specifying patient dose from DXA investigations as it relates directly to the radiation risk involved. The effective dose for hip and spine scans performed on the QDR Discovery 4500 was 7.5 μSv using the express scan mode. This is the equivalent of 1 day or less of natural background radiation exposure. The effective dose for whole body DXA scans was 4.2 μSv .

2.8.1 Measurement of BMD at the lumbar spine

Each subject was positioned supine on the DXA scanning table, with legs elevated on to a foam block at 90° (Figure 2.4). This position reduces lumbar lordosis and keeps both femora aligned and flat on the table, enhancing the visibility of the lumbar vertebrae for accurate image analysis (Figure 2.4). To ensure that the fifth to first lumbar vertebra (L1-L5) was visible in the field of view (FoV), the scan position was initiated at the midline at approximately 1 inch below the iliac crest (Figure 2.5). Each scan acquisition was performed on express mode; however, for subjects over 80kg this was performed on array mode.

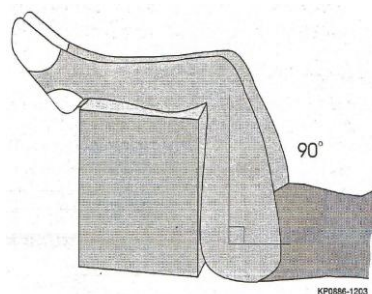


Figure 2.4 Demonstrating correct positioning required for an AP lumbar spine. Modified from the Hologic QDR 4500 Discovery Operator Manual (www.hologic.com).

The completed scan image was analysed using the Discovery QDR Apex image analysis software. A rectangular region of interest (ROI) was placed from L1- L4 and intervertebral lines were then placed between L1-L4, dividing each vertebra into a separate region of interest (Figure 2.6). The image analysis results were plotted against the reference data (NHANES III) [548] to determine the overall T and Z-score for the subject.

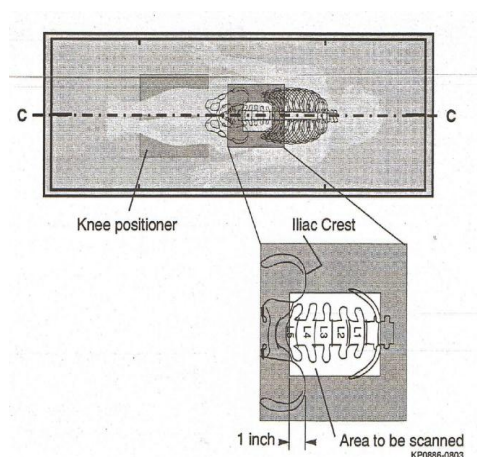


Figure 2.5 demonstrating the correct positioning on the scanning couch for an AP lumbar spine scan. Modified from the Hologic QDR 4500 Discovery Operator Manual (www.hologic.com).

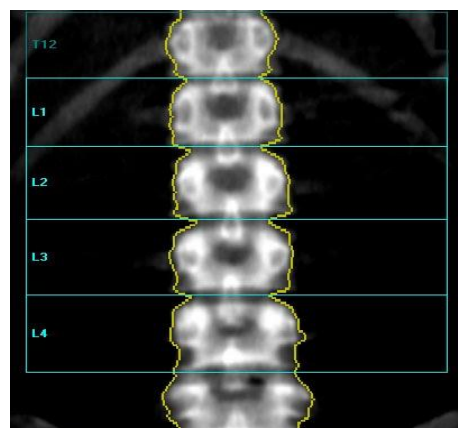


Figure 2.6 DXA scan of the lumbar spine L1-L4. Accessed 2010 (www.hologic.com).

2.8.2 Measurement of BMD at the Hip

All hip scans were performed preferably using the left hip for accessibility. However, subjects with metal prosthesis including hip replacements on the left hand side had DXA scans performed using the right hip. The subject was positioned supine on the scanning table with legs straight. The left leg was internally rotated and abducted in order to separate the femoral neck from the ischium and the foot secured to a positioning aid to prevent outward rotation (Figure 2.7); the rotation was such that the lesser trochanter of the femur was not visualised on the image (Figure 2.7). Scan acquisition started at the midline of the femur, approximately 1.5 inches below the greater trochanter (Figure 2.7) and performed on express mode. Image analysis was performed using the QDR Apex image analysis software. A ROI box was placed around the hip as shown in Figure 2.8. The midline was positioned along the axis of the femoral neck and the femoral neck box was positioned across the femoral neck, close to the greater trochanter (Figure 2.8). As with the lumbar spine analysis, the results were plotted against the reference data (NHANES III) [548] to determine the total hip T and Z-scores for the subject, and T and Z-scores for the femoral neck.

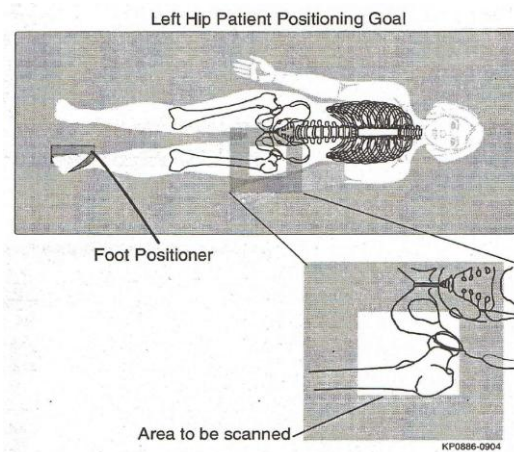


Figure 2.7 demonstrating the correcting positioning on the scan couch for the hip scan, with correct foot positioning. Modified from the Hologic QDR 4500 Discovery Operator Manual (www.hologic.com).

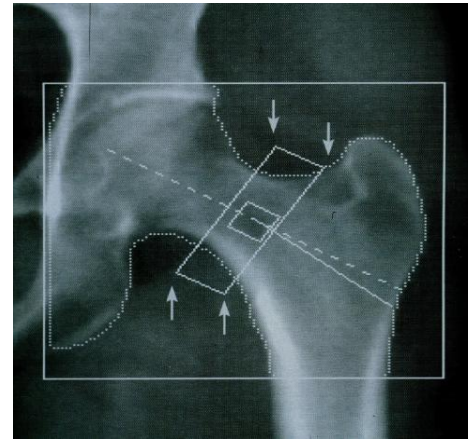


Figure 2.8 DXA scan of the proximal femur (left hip) showing ROI placement for image analysis. The femoral neck box is indicated by the arrow.

2.8.3 Whole body DXA

Each of the study subjects had whole body DXA scans performed to measure body composition and whole body BMD/BMC. The scan provided information on lean and fat mass in grams and fat percentages. Whole body composition scans were acquired with the subject lying supine on the DXA scanning table with the head positioned straight and arms placed palm down either side. Legs were straight but slightly separated with toes touching as shown in Figure 2.9. The regions of interest were automatically selected by the Discovery QDR Apex imaging software for body composition analysis within each of the anatomical regions (Figure 2.9).

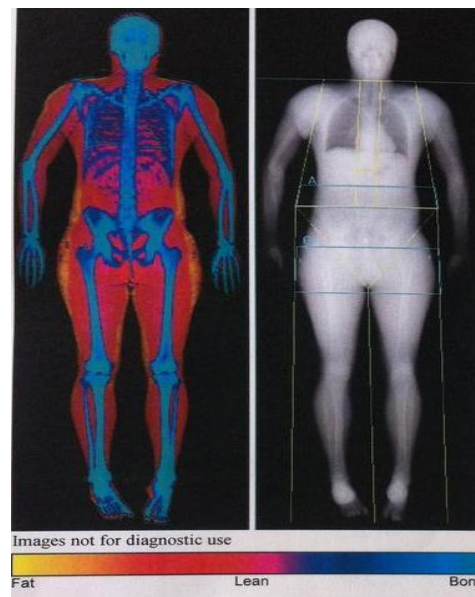


Figure 2.9 Whole body DXA scan showing regions of interest automatically selected by the image analysis software in order to establish fat and muscles parameters within those regions.

2.9 Lateral Vertebral Fracture Assessment (VFA) scans

In this study Lateral VFA scans were performed for the purpose of quantifying AAC. Traditionally lateral VFA scans are used to assess vertebral deformities. However, the anatomical positioning of the AA anterior to the lumbar spine allowed for the assessment of calcium deposits within the abdominal aortic region. The scans in the present study were limited to the lumbar region only (L1-L4). Calcified deposits detected using lateral VFA scans have a similar x-ray attenuation to bone, and lateral VFA scans can provide a low radiation dose alternative method for the semi-quantitative assessment of AAC (Figure 2.10). All lateral VFA scans were performed using the Hologic High Definition Instant Vertebral Assessment (IVA-HD) which performs a single-energy scan of the lumbar spine (L1- L4) in 15 seconds with a radiation dose of $<10\mu\text{Sv}$ [547].

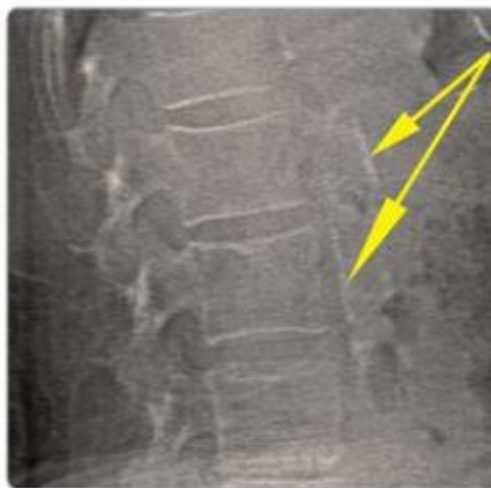


Figure 2.10 calcification of the anterior and posterior aspect of the abdominal aortic walls anterior to the lumbar vertebra. Calcification is marked by yellow arrows at the anterior and posterior walls. Modified from Hologic.com.

Lateral VFA scans were acquired with the subject lying supine on the scanning table with legs elevated up on a foam block at 90° to reduce lordosis (Figure 2.4) following the same principle as for the AP lumbar spine scan. However, for successful image acquisition the arms were held in position above the head with a foam contour pillow to prevent them from obstructing the SFoV (Figure 2.11).

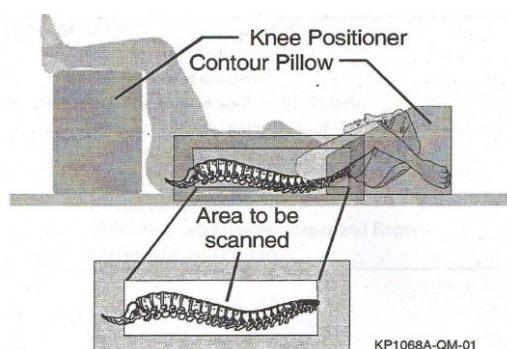


Figure 2.11 demonstrating the correct positioning for the lateral VFA scan acquisition. Modified from the Hologic QDR 4500 Discovery Operator Manual (www.hologic.com).

All scans were reviewed using the Hologic QDR Apex software system to determine the presence of calcification within the AA (Figure 2.10). The 24- and 8-point AAC scores were used to grade the amount of calcification, as described by Schousboe [549].

2.9.1 24-Point scoring method for quantifying AAC

Calcification as detected using lateral VFA scan images was quantified using a previously validated semi-quantitative 24-point scoring method [550]. With this method, scans were assessed for the presence of calcification in the AA within the regions corresponding to the first through to fourth lumbar vertebrae. The anterior and posterior aortic walls were visually segmented into four sections corresponding to areas in front of L1-L4 using the midpoint of the inter-vertebral space (Figure 2.12). The severity of the anterior and posterior AC was graded individually on a 0-3 scale for each lumbar segment (Table 2.2). Aortic calcification was scored as 0 if there was zero calcification, as 1 if one-third or less of the aortic wall in a segment was calcified, as 2 if more than one-third but two-thirds or less of the aortic wall was calcified or as 3 if more than two-thirds of the aortic wall was calcified. Scores ranged from 0 to 6 for each vertebral level. The results for all 4 lumbar vertebra, including scores for both anterior and posterior walls, were summed to provide one score for each subject. The total score range was from 0 to 24.

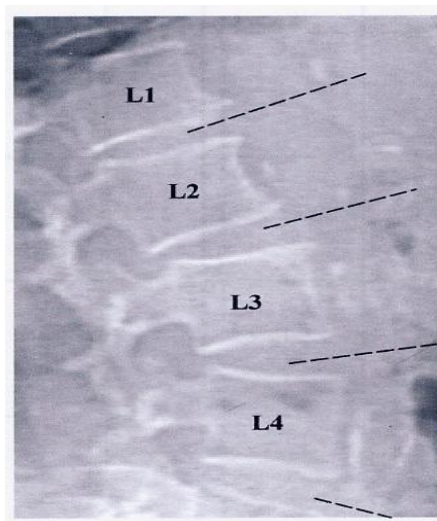


Figure 2.12 Example of a VFA scan showing the AA. The aorta has been visually segmented at regions corresponding to each of the lumbar vertebrae. L2, L3 and L4 show calcification at the anterior and posterior walls for the aorta.

Description	Score
No calcification seen	0
Deposit < one- third length of aortic wall	1
Deposits > one-third but < two-thirds the length of aortic wall	2
Deposits > two-thirds the length of aortic wall	3

Table 2.2 the 24-point calcium scoring method as described by *Schousboe* [549].

2.9.2 8-point scoring method for quantifying AAC

The 8-point score, a simplified version of the 24-point AAC scoring method, was developed by Schousboe, Wilson, and Kiel [549, 550], and examines only the anterior and posterior aspects of the aortic wall adjacent to vertebrae L1-L4. The 8-point score is the sum of the total length of calcification for each of the anterior and posterior aortic walls in front of vertebrae L1 to L4. A score of 0 is given for zero calcification, 1 if the aggregate length of calcification is less than or equal to 1 vertebral height, 2 if that length is greater than 1 but less than or equal to 2 vertebral heights, 3 if that length is greater than 2 but equal to or less than 3 vertebral heights, and 4 if the aggregate length of calcification is greater than 3 vertebral heights (Table 2.3). The total score range was 0 to 8.

Description	Score
No calcification seen	0
Aggregate length of calcification = height of one vertebra	1
Aggregate length of calcification > one but = two vertebra	2
Aggregate length of calcification > two but = three vertebra	3
Aggregate length of calcification > three vertebra	4

Table 2.3 8-point calcium scoring method as described by *Schousboe et al* [549, 550].

2.10 Pulse Wave Velocity (PWV)

Pulse wave velocity measurements were obtained using the SphygmoCor (AtCor Medical LTD, Australia) PWV system. The system used ECG and an ultrasound tonometer to measure the pressure pulse waveform sequentially in two peripheral artery sites - carotid & femoral. This method is non-invasive and the measurements obtained in part, rely on the degree of atherosclerotic burden within the aorta. During each cardiac cycle the pulse

wave travels from the heart down the arterial wall in advance of blood flow. The more rigid the wall of the artery, the faster the wave moves (Figure 2.13).

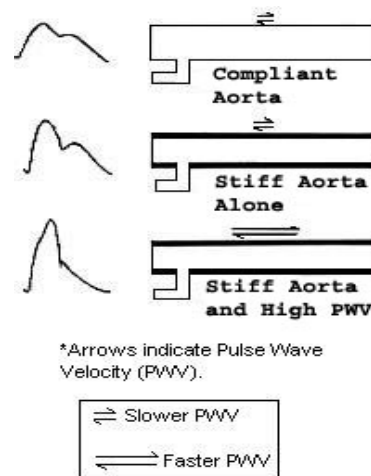


Figure 2.13 Pulse wave velocity and the aortic pressure waveform. Accessed 2012.

The pulse pressure waveform was recorded simultaneously with the aid of an ECG signal, which provided an R-wave timing reference. Pulse pressure recordings were performed consecutively at the carotid and femoral arterial sites using a pressure tonometer which was used to transcutaneously record the pressure pulse waveforms in the underlying artery. The carotid and femoral arteries were used so the PWV could be measured in the section of artery that included the aorta. In the present study each subject was required to lie supine on an examination couch, exposing the areas required to attach the ECG electrodes and also the femoral region (Figure 2.14). An electronic blood pressure monitor (Omron) was used to determine systolic and diastolic blood pressures, which were entered onto the computer system. The entered values calibrated the averaged aortic waveform. Measurement of the PWV distance was estimated by measuring from the sternal notch to the femoral artery and this value (mm) was entered into the system. To obtain the PWV measurement, the tonometer was placed on the carotid and femoral arterial sites to record the pulse pressure waves (Figure 2.15). The transit time was determined from the time delay between the two corresponding systolic foot waves of the two signals. The distance travelled was estimated from the distance between the two sites (Figure 2.16). To ensure an adequate reading, PWV was averaged over 10 consecutive cardiac cycles. The procedure was repeated 3 times in order to calculate an average PWV for each subject. The validation of this method and its reproducibility has been previously described by Asmar et al, with intra- and inter-observer repeatability coefficients of 0.935 m/s and 0.890m/s respectively [337].

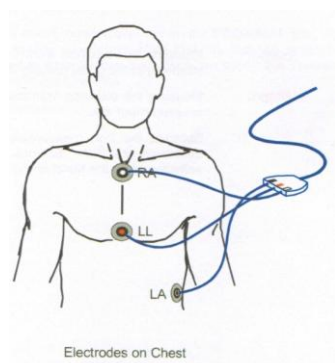


Figure 2.14 showing the correct ECG electrode placements for carotid-femoral PWV measurement. Modified from the SphygmoCor Pulse Wave Velocity System Operator Manual (www.atcormedical.com)

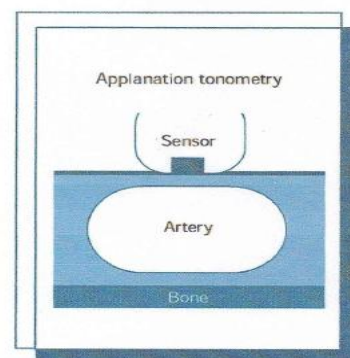


Figure 2.15 The principle of applanation tonometry. The artery is partially compressed against a hard structure. The small (0.5mm in diameter) sensor detects the force of the artery wall. Accessed 2012 (qjmed.oxfordjournals.org).

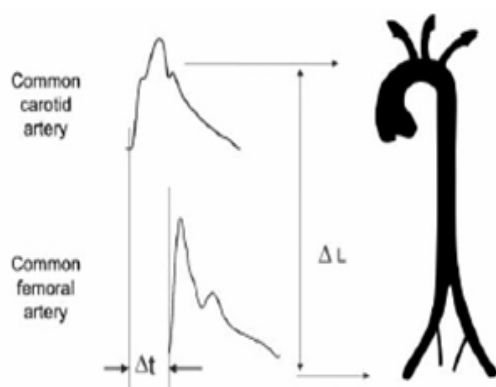


Figure 2.16 Calculation of PWV - The time delay between two foot waves at peripheral artery sites. $D/\Delta t$ (cm/s). Accessed 2012 (www.indiana.edu).

Aortic PWV is measured as:

$$\text{Aortic PWV} = \frac{\text{Distance m/s}}{\text{Transit time}}$$

2.11 Computed Tomography (CT)

The Philips Precedence single-photon emission computed tomography (SPECT)/CT 16-Slice multi-detector (MDCT) system (Philips Healthcare, Best, Netherlands) was used for scans of the chest and abdomen (Figure 2.17). The scanner is capable of performing non-diagnostic, unenhanced CT for the purpose of quantifying VC. The precedence SPECT/CT performs Spiral/helical scanning which is characterised by a continuous gantry rotation and continuous data acquisition while the scan couch is moving at constant speed. In the present study a scan field of view (SFoV) of 50cm was required to include both the chest and abdomen incorporating the carotid to the femoral arteries. The camera has a rotation speed of 0.5s. The radiation dose for this scan was 5mSv; moderately lower

than a diagnostic CT scan used in routine clinical practice with radiation doses ranging between 10 – 15mSv.

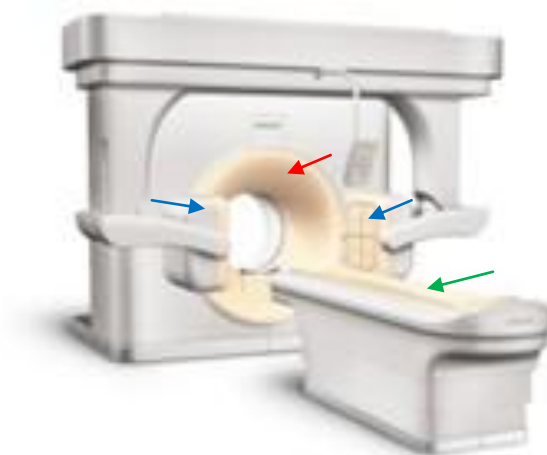


Figure 2.17 The Philips Precedence 16-slice MDCT SPECT/CT system (Philips Healthcare, Best, Netherlands), showing the rotating gantry (red arrow), X-ray source (blue arrows), and scan couch (green arrow). Modified from healthcare.philips.com.

Computed tomography utilises computer-processed X-rays to produce tomographic images or 'slices' of specific areas of the body. Digital geometry processing was used to generate a three-dimensional image of the inside of the subject from a large series of two-dimensional X-ray images taken around a single axis of rotation. Computed tomography produces a volume of data that can be manipulated, through a process known as windowing, in order to demonstrate various bodily structures based on their ability to block the X-ray beam or attenuation. Once the scan data had been acquired, it was then processed using a form of tomographic reconstruction, providing a series of transaxial images which were then reconstructed to contiguous 3mm slices. The final scan image was made up of pixels, which are 2-dimensional units based on the matrix size and field of view. When the CT slice thickness was also factored in, the unit was known as a voxel, which is a three-dimensional unit. The voxels in the image obtained were displayed in terms of relative radio density. The voxel itself was displayed according to the mean attenuation of the corresponding tissue on a scale from +3071 being the most attenuating to -1024 or the least attenuating on the Hounsfield scale. Images were sent to the JETStream workspace software which was developed specifically for SPECT/CT to enable image reconstruction, manipulation and analysis. The image data was then sent to the Hermes Gold™ software for image analysis and the quantification of abdominal aortic and VC.

In the present study each subject was required to remove any metal that may be in the SFOV and put on a hospital gown. The subject was then required to lay supine on the scan bed with arm raised above their head, and legs slightly elevated on a foam knee rest. To ensure all required vascular sites were within the FoV, a surview scan was performed initially from under the angle of the mandible to the proximal third of the femur. Instructions were provided by the technologist prior to commencing the scan, on how to perform a breath hold for 15 seconds during the scan image acquisition. The breath hold was important; scans for coronary imaging traditionally use ECG gating to reduce motion artefacts on the image. As ECG gating was not used during the scan acquisition, the breath hold served to reduce movement thus minimising the risk of motion artefacts on the scan images. Subjects from the preliminary imaging study sub-set (Section 2.5.1) were required to get off and back on the scanner for a duplicate scan using the same scan protocol. X-ray slice data was generated using an X-ray source that rotated around the subject, allowing numerous data scans to be progressively taken as the subject was gradually passed through the gantry (Figure 2.17).

2.11.1 CT Image analysis

The Hermes Gold TM image analysis software provides automatic or manual transfer from Digital Imaging and Communications in Medicine (DICOM) supported CT systems and NM Gamma Cameras such as the Philips Precedence SPECT/CT camera, for local storage or automatic forwarding to other systems such as the picture archiving and communication system (PACS).

The scan image was first located on the Hermes Gold browser from the scan storage archives for Guy's Hospital using the subject study number. For subjects that had repeat scans, images were labelled as scan 1 and scan 2 to enable the reader to distinguish between the first and second scan. The image was opened in a 'hybrid viewer' mode enabling the viewer to scroll through each of the transaxial slices and identify anatomical regions of interest. The image slice numbers were obtained and each of the anatomical regions was recorded on an 'anatomical region data sheet' that had been developed for this study. Each 3mm scan slice was analysed individually and calcified deposits were defined as any area greater than 1mm^2 with an attenuation coefficient of ≥ 130 Hounsfield units (HU). Calcification within an ROI was automatically selected by a thresholding function. For this study an attenuation threshold of 130 Hounsfield Units (HU) had been set, in keeping with other studies. Calcified deposits were labelled to emphasise vascular sites using anatomical location and number; for example a calcified deposit located in the AA was labelled as AAORTA Dep 1. This process was repeated for each transaxial slice

and provided a volume measurement of calcification for all contiguous slices. Once image analysis was complete, the data file with information pertaining to the amount of calcification identified was then anonymised and exported for statistical analysis.

2.12 Methods used to quantify calcification using CT

2.12.1 The Agatston score

The Agatston scoring method has been widely used [541-546, 551, 552] and is a validated and commonly used method for quantifying coronary calcium. The method is based primarily on the maximum CT number (measured in Hounsfield Units [HU]) or x-ray attenuation coefficient, and the area of the calcium deposit. Score values have been established on the basis of measurements from electron beam computed tomography (EBCT) to establish clinical risk of future CV events. The score helps to determine if a subject has atherosclerosis or not and the extent of the disease. The calcified lesions are firstly identified on CT images by the rater (Figure 2.18) by applying a threshold of 130HU. Tissues with densities equal to or greater than 130HU are considered to correspond to echogenic calcium. For each artery in question a region of interest (ROI) is drawn around each calcified lesion. The maximum CT number of the ROI is determined and used to assign a weighting factor. The area of the ROI is also determined and the Agatston score is computed as the product of the weighting factor and the areas. This score does not correspond to a physical measure; the Agatston score relies on a weighting factor to provide the calcium score. A calcified lesion is defined as an area of 0.5mm^2 or greater and the weighting factor assigned is dictated by the maximum CT number for the lesion. The sum of all lesion scores is used to generate the total calcium score (TCS).

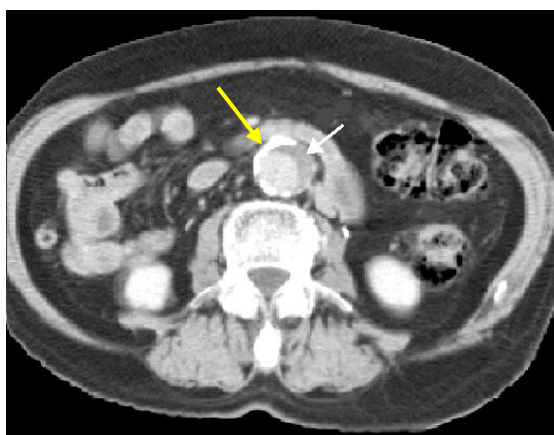


Figure 2.18 shows an axial scan showing the aorta (white arrow) and calcification within the wall of the aorta (yellow arrow). Accessed 2011 (*Medscape.com*).

Score of calcified deposit (CS_i) = Area of deposit (A_i) x weighting factor (w_i)

Where:

$$CS_i = A_i \times w_i$$

Weighting factors (w_i)

$$1 = 130 - 199 \text{ HU}$$

$$2 = 200 - 299 \text{ HU}$$

$$3 = 300 - 399 \text{ HU}$$

$$4 = >400 \text{ HU}$$

This equation is applied to each deposit found in each transaxial image.

Equation 2.1

2.12.2 Modified Agatston score

A modified version of the Agatston score was developed to improve sensitivity and specificity, using the mean attenuation coefficient or CT number rather than the maximum [553]. The modified Agatston score (AS_m) is calculated as the product of the mean attenuation for all pixels in the deposit with attenuation higher than 130 HU (CT_{mean}) and the volume of the calcified deposit (V):

Where:

$$AS_m = CT_{mean} \cdot V.$$

AS_m = modified Agatston score

CT_{mean} = mean CT number of deposit

V = volume of deposit

Equation 2.2

2.12.3 Volume score

The volume score was first described by Callister et al in 1998 [554]. It was initially designed to resolve the issue of slice thickness and spacing by computing a volume above threshold. The calcium volume can be simply calculated by multiplying the number of voxels (V_n) with the voxel volume (V_v) in a deposit:

$$V = V_n \times V_v$$

Where:

V = Voxel

V_n = Number of voxels

V_v = Volume of one voxel

Equation 2.3

2.13 Anthropometric measurements, blood pressure and medical history

All study subjects had their height and weight measured to the nearest 0.1m and 0.1kg respectively. Body mass index (BMI) was then calculated as weight (kg) divided by height squared (m^2). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) was measured supine using an electronic oscillometric blood pressure device (Omron, Japan) after the subject had rested for a minimum period of 15 minutes. Mean arterial pressure was calculated as $[(2 \times \text{DBP}) + \text{SBP}]/3$. A detailed medical history was recorded for each subject, obtained using questionnaires and by personal interviews. Information collected included history of CV events including myocardial infarction (MI), angina, trans-ischemic attack (TIA), peripheral arterial disease (PAD), and intermittent claudication (IC), the confirmed diagnosis of diabetes mellitus, hypertension, and hypercholesterolemia, fracture history, smoking habits, alcohol intake and physical activity levels, and family history of CVD and low trauma fractures. Information was collected regarding the use of prescribed and over-the-counter preparations including but not exclusively, BPs, antihypertensive medications, analgesia and anti-inflammatory drugs, diuretics and vitamin and mineral supplementation.

2.14 Laboratory assessments

At each study visit, all subjects had venous blood samples collected by a clinical scientist (SE) following a 10 hour overnight fast. Thirty millilitres of whole blood was collected in serum separation tubes (SST's) for measurement of lipid and bone profiles. The prospective study group had also had routine haematology and clinical chemistry tests performed at screening to exclude any secondary causes of OP. These included a full blood count (FBC), erythrocyte sedimentation rate (ESR), protein electrophoresis, parathyroid hormone (PTH), vitamin D and a full lipid, bone, renal and thyroid profile. Six millilitres of whole blood was sent to St Thomas Hospital Department of Clinical Chemistry for the measurement of total cholesterol, triglycerides, HDL and LDL-cholesterol levels in addition to calcium, albumin corrected calcium, albumin, phosphate and sodium, magnesium and chloride using standard laboratory methods on the Roche Modular analysers (Roche Diagnostics Limited, West Sussex, UK). The remaining 24mls of whole blood was centrifuged and serum was separated into 10 aliquots which were then frozen at -70° for the analysis of systemic and local factors involved in bone remodelling and atherosclerosis. These included PTH, PINP, CTX, vitamin D, sclerostin and Dkk1. Parathyroid hormone and vitamin D was analysed by consultant chemical pathologist Dr Geeta Hampson in the Department of Clinical Chemistry at St Thomas' Hospital. PINP, CTX, sclerostin and Dkk1 were analysed by the commercial bone marker laboratory at the

University of Sheffield. During the prospective study a further blood sample was collected at 6, 12 and 24-months for the measurement of circulating endothelial progenitor cells (EPCs). These samples were processed immediately after collection and analysed using flow-cytometry to enable identification and characterisation of EPCs. Analysis was performed by Dr Ulrike Mödder in the flow-cytometry department of the Clinical Trials Unit at Guy's Hospital.

Chapter 3

The intra- and inter-rater agreement of computed tomography and lateral VFA images for the quantification of aortic calcification

3.1 Introduction

Recent evidence has shown age-independent associations between OP and CVD, which can be explained in part by an increase in VC seen in patients with OP [316, 360, 472]. From the sixth decade onwards, most individuals will have evidence of progressively large calcium deposits within the major arteries [555] which lead to decreased arterial elasticity and impaired CV function, resulting in an increased risk of CV events and significant morbidity and mortality [491, 556, 557]. Due to its good precision and accuracy, non-contrast CT is recognised as the gold-standard method for quantifying AC [558-561]. However, CT is an imaging method limited by a lack of availability, high radiation exposure and high cost [562, 563]. Alternatively, bone densitometry, a low radiation and low cost imaging method used to routinely assess BMD and vertebral deformity may provide a good substitute to CT for quantifying AC [316, 564]. Anatomically, the AA is located anterior to the lumbar spine and therefore can be visualised on lateral VFA scans and this technique has been shown to be a useful imaging method for the qualitative and semi-quantitative assessment of AC [564]. Previous studies on high risk populations with CKD that have a high incidence and extent of VC [558, 559, 561, 565, 566] and more recently, a low risk healthy population [567] have reported good sensitivity of lateral DXA comparable to CT for the assessment of AAC [558, 561, 567]. Abdominal aortic calcifications detected by conventional radiographs, which are often performed in clinical trials of patients with OP, have also been shown to be a significant predictor of overall CV disease incidence and mortality [555]. A study by Schousboe et al confirmed a close correlation between AAC assessed using lateral VFA and radiographic AAC [564]. The results from these studies demonstrate that lateral DXA imaging, used primarily for the identification of prevalent vertebral fracture, also allows a simultaneous assessment of AAC; an important CV disease risk factor commonly encountered in postmenopausal women referred for bone density screening [564]. There is limited information in the literature regarding the intra- and inter-rater agreement and test-retest reproducibility of non-contrast CT for quantifying VC at non-coronary sites, with data limited to high risk populations with impaired renal function, which may not represent results obtained in a study of healthy postmenopausal population [558]. Moreover, there is limited information

regarding the reproducibility of calcium scoring methods, including the Agatston, modified Agatston and volume scores for quantifying VC at non-coronary sites using CT. A study by Bowden et al evaluated the inter-rater agreement for measuring AAC using CT, but the study was limited by its small sample size and quantified calcification using only the volume score. Furthermore, the study did not evaluate intra-rater agreement and test-retest reproducibility, and image analysis was restricted to only a small 4.95cm section of AA [568].

Previous studies have assessed the intra- and inter-rater agreement of lateral VFA for quantifying AAC using both the 24-point and 8-point scoring methods but these studies have again been limited to high risk populations with CKD [558, 569]. The evaluation of both the intra- and inter-rater agreement and test-retest reproducibility of moderate dose CT for quantifying VC at non-coronary vascular sites and the intra- and inter-rater agreement and test-retest reproducibility of lateral VFA will provide valuable data on the reliability of both methods for future research studies and potential implementation into clinical practice. A summary of the studies used to compare and correlate with the results from the present study are presented in Table 3.1.

Table 3.1 Overview of previous studies evaluating the agreement of imaging methods lateral VFA and CT for the measurement of AC and VC and the scoring methods used to quantify AC/VC.

Author (year)	Study design	N	Population	Mean age	Imaging method	Calcium scoring method
Wilson (2001) ^[339]	Follow-up	2515	GP	>60	Rad	24-Point
Schousboe (2006) ^[570]	Cohort	57	PM	>65	DXA	8-Point
Schousboe (2007) ^[564]	Cohort	174	PM	68.7	Rad	24-Point
Bowden (2009) ^[568]	Cohort	34	GP	NP	CT	Volume
Toussaint (2009) ^[569]	Cohort	40	HD	58.5	DXA	8-Point
Toussaint (2010) ^[558]	Cohort	44	CKD	58.5	CT	24-Point
Cecelja (2011) ^[571]	Cohort	40	GP	>60	CT	Agatston
						Volume

PM, postmenopausal; HD, haemodialysis; GP, general population; CKD, chronic kidney disease; CT, computed tomography; DXA, dual-energy x-ray absorptiometry; SXA, single-energy x-ray absorptiometry; Rad, radiographs.

3.1.1 Study aims

The aim of this part of the study was to evaluate the intra- and inter-rater agreement of CT for quantifying VC and to examine the test-retest reproducibility of three scoring methods, including the Agatston, modified Agatston and volume scores, for quantifying VC in multiple large vessels using CT. The use of moderate dose, non-contrast CT may provide a reliable method for quantifying VC within the AA and at other vascular sites for use in future research studies. Furthermore, the current study also examined the intra- and inter-rater agreement and test-retest reproducibility of lateral VFA for quantifying AAC using the previously validated 24-point and 8-point scoring methods. Results from this part of the study provide valuable data for assessing the potential role of lateral VFA scan images as a quick and simple method of assessing AC in women with low BMD and OP in both research studies and routine clinical practice.

3.2 Study population

The study population consisted of 63 healthy, ambulatory postmenopausal women with a mean age of 61 years who had attended the Osteoporosis Unit to take part in a large cross-sectional study as previously described in Section 2.4, Chapter 2. All subjects had CT scans of the chest and abdomen to quantify calcification at multiple vascular sites and lateral VFA scans of the lumbar spine to quantify AAC. A sub-set of 8 subjects had duplicate CT scan measurements at one session to assess test-retest reproducibility following confirmation of AAC on lateral VFA scans. Only women aged 65 and over were selected for the sub-set in an attempt to avoid subjects with zero or minimal amounts of VC. The number of women selected for duplicate CT scans was restricted because of ethical issues associated with the radiation dose involved. A sub-set of 19 women had duplicate lateral VFA scans at one session to assess test-retest reproducibility.

3.3 Materials and Methods

3.3.1 CT imaging

Thirty-three of the 63 subjects underwent non-contrast non-diagnostic, un-gated CT scans of the chest and abdomen. The scans were performed using the Philips Precedence 16-slice SPECT/CT helical scanner (Philips Healthcare, Best, Netherlands) as described in Section 2.11, Chapter 2. The scan field of view (FoV) was approximately 50cm which included the iliac arteries (IA), abdominal aorta (AA), thoracic aorta (TA) and carotid arteries (CA). In order to measure test-retest reproducibility, the first 8 subjects had repeat scans which involved getting off and back on the scanner for a second scan immediately

following the first scan, using the same scan acquisition protocol for the first and second scans (Section 2.11.1, Chapter 2). Scan images were reconstructed into contiguous 3mm transverse slices for viewing on the image analysis workstation (Figure 3.1), and each scan consisted of a median of 170 slices, varying with subject height. All images were viewed and analysed using the semi-automated hybrid viewer function on Hermes Gold TM imaging software.

3.3.2 Quantitative assessment of calcification on CT

Scans were assessed by two raters (a clinical scientist [SE] and a Consultant Radiologist [DD]) who were blinded to subject demographics, BMD results, lateral VFA scores and to each other's readings. Scan images were identified by the subject's study number only. Scans were labelled as scan 1 and scan 2 on the workstation to enable the viewer to distinguish the first from the second scan. Each 3mm transverse slice was analysed individually and calcified deposits were defined as any area greater than 1mm^2 within the blood vessel being assessed with an attenuation coefficient of ≥ 130 Hounsfield units (HU). Individual calcified deposits on each transverse CT slice were identified by the rater and then a 'seeding' tool was used to delineate each deposit using the thresholding method as described in Chapter 2. Calcified deposits were labelled according to anatomical location and deposit number; for example a calcified deposit located in the AA was labelled as AAORTA Dep 1 (Figure 3.2 and 3.3). The total Agatston [349], modified Agatston [553] and volume scores [554] were calculated by summing the scores obtained for all cross-sectional slices (Section 2.12, Chapter 2). Each scan was analysed twice by each rater with a minimum of two weeks between the first and second analysis, with each scan taking approximately 45 mins to analyse.

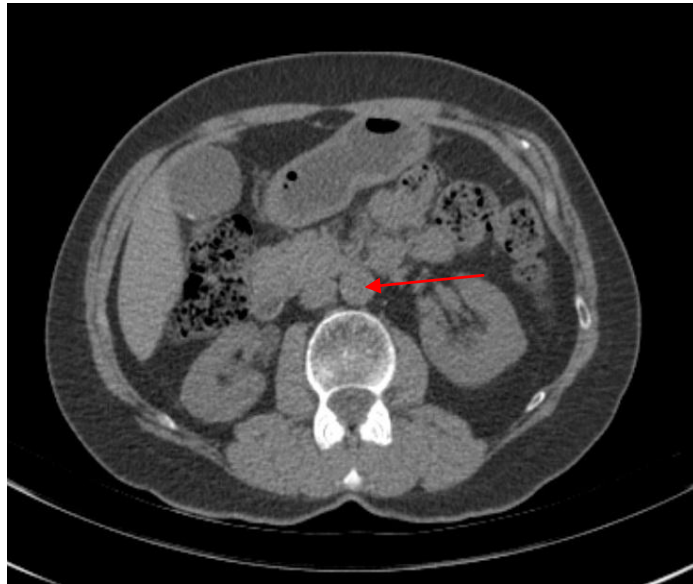


Figure 3.1 Transaxial CT image showing normal aortic anatomy with no calcified deposits in the aorta (red arrow) located anterior to lumbar vertebral body L2.

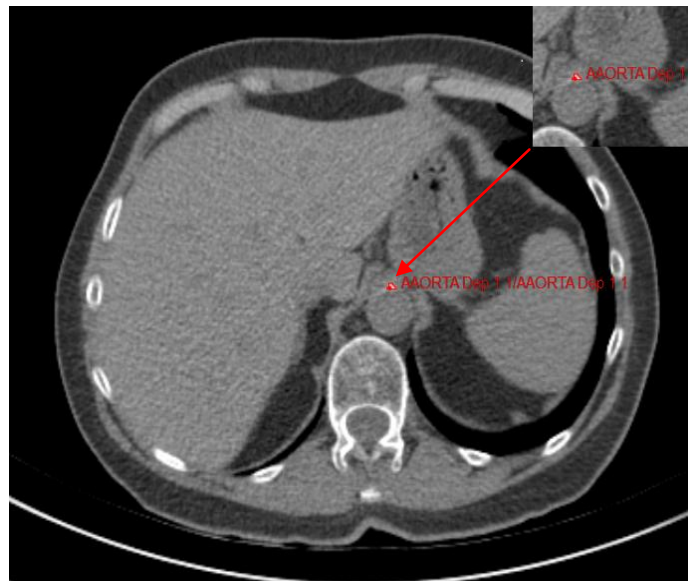


Figure 3.2 Transaxial CT image showing a calcified deposit within the abdominal aorta with a ROI around the calcified deposit (shown in red). Calcium scores for this deposit were: Agatston 20 AU, modified Agatston score 472 AU and volume score 0.017cm³.

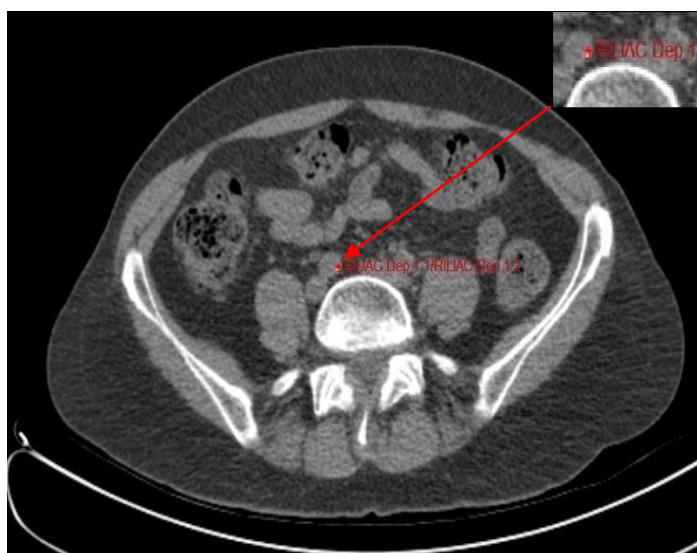


Figure 3.3 Transaxial CT image showing a calcified deposit within the right iliac artery (red arrow) with a ROI around a calcified deposit (shown in red). Calcium scores for this deposit were: Agatston score 28 AU, modified Agatston score 686.6 AU and volume score 0.021cm^3

3.3.3 Lateral VFA scans

Thirty of the 63 subjects had single-energy lateral VFA (IVA-HD) scans of the lumbar spine using the Hologic Discovery QDR 4500 densitometer (Hologic, Bedford MA) as described in Section 2.9, Chapter 2. Scans were obtained using an established local protocol for lateral VFA scan acquisition which was modified to include the lumbar spine region only. Scans were acquired using a rotating C-arm which required the subject to lie supine with arms raised. In order to measure the test-retest reproducibility of lateral VFA for quantifying AAC, a sub-set of 19 subjects had repeat scans which required getting off and back on the scanner for a second scan immediately following the first scan, using the same scan acquisition protocol for the first and second scans.

3.3.4 Semi-quantitative assessment of AAC

Abdominal aortic calcification was assessed on the lateral VFA scan images by two raters (both clinical scientists [SE and MF]) both blinded to subject demographics, BMD results and to each other's scores. Scans were identified only by subject number and each scan was analysed twice by each rater with a minimum of two weeks between the first and second image analysis. Abdominal aortic calcification was evaluated using the previously described semi-quantitative 24-point and 8-point scoring methods (Sections 2.9.1 and 2.9.2, Chapter 2) in which both the location and the severity of calcification at each lumbar

vertebral segment (L1–L4) were evaluated [564]. Figure 3.4 illustrates the 24-point scoring method as described in Section 2.9.1, Chapter 2. The analysis of lateral FVA scan images took somewhere in the region of 7-10 minutes, with minimal difference in time taken between the 24- and 8-point scoring methods.

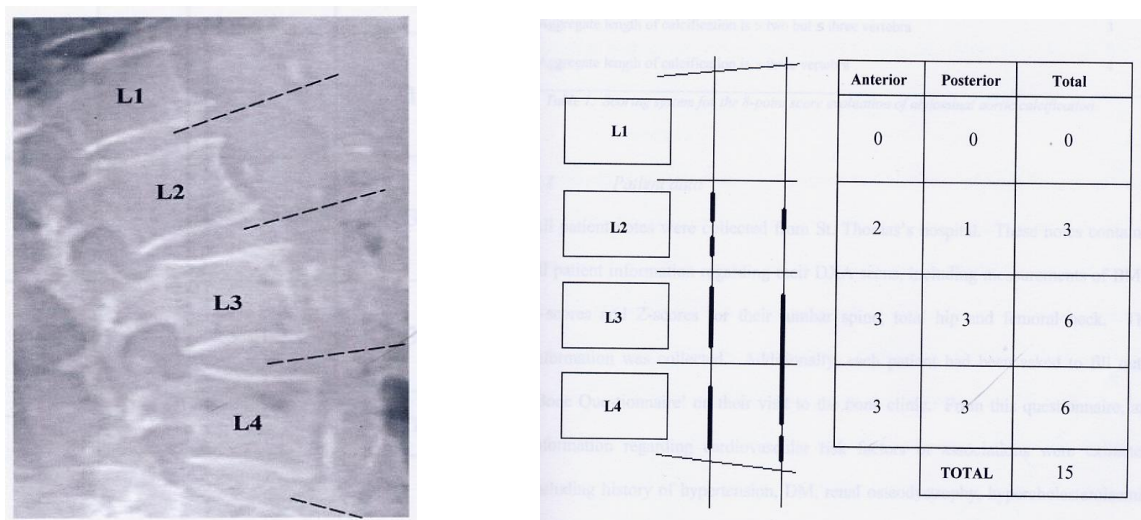


Figure 3.4 Example of a lateral VFA scan showing calcification within the anterior and posterior walls of the AA. The aorta is visually segmented to correspond with vertebrae L1-L4. The example grid on the right hand side illustrates the anterior and posterior aspects of the aortic wall in relation to the lumbar spine. The 24-point score, as described in Section 2.9.1, Chapter 2, is applied to correspond to the location and extent of calcification identified within the segmented aorta.

3.4 Statistical analysis

The study population characteristics are presented as the mean and standard deviation (SD) unless otherwise stated. The Students t-test and chi square test was used to test for significant differences in characteristics between the total study cohort of 63 postmenopausal women and sub-groups. Intraclass correlation coefficients (ICC) were calculated to assess intra- and inter-rater agreement and test-retest reproducibility for each of the CT and lateral VFA scoring methods. Bland-Altman plots were used to visually assess intra- and inter-rater and test-retest variability and the bias, percentage difference and limits of agreement between readings, raters and tests calculated. Bland-Altman plots provide a graphical statistical tool to compare two sets of readings, and allow the degree of variation between each reading to be analysed objectively [572].

3.5 Results

The study population characteristics are shown in Table 3.2. All subjects were female with a mean age of 61 years (range 50 to 81 years) with no prior history of CV events. Thirty-five per cent of subjects had a family history of CVD and 13 per cent were taking prescribed calcium and vitamin D supplementation at the time of the investigations. Statistical differences in characteristics were observed between the total study cohort and those in the sub groups (Table 3.2). The CT sub-study group subjects were significantly older than the total study population and had lower total hip and femoral neck T-scores. Subjects in both the lateral VFA and lateral VFA test-retest groups had a significantly higher average femoral neck T-score.

Table 3.2. Study group characteristics.

	Study cohort	CT study	CT test-retest sub-study	Lateral VFA study	Lateral VFA test-retest sub-study
	63	33	8	30	19
Age (years)	61.2 (7.0)	62.8 (8.2)	75.7 (3.2) ^c	59.5 (5.0)	59.8 (3.5)
BMI (kg/m ²)	25.5 (4.5)	25.2 (4.7)	28.8 (5.4)	25.7 (4.2)	26.5 (4.4)
Lumbar spine T score	-1.4 (1.4)	-1.8 (1.6)	0.0 (2.4)	-1.0 (1.0)	-0.9 (1.1)
Total hip T score	-0.8 (0.8)	-1.2 (0.7) ^b	-0.7 (0.8)	-0.5 (0.8)	-0.4 (0.9)
Femoral neck T-score	-1.3 (0.9)	-1.7 (0.8) ^b	0.7 (0.1)	-0.8 (0.9) ^a	-0.7 (0.9) ^a
History of fracture (%)	41	22	6	19	18
Current Smoker (%)	8	6	2	2	0
Antihypertensive meds (%)	11	15	38	7	5
Osteoporosis therapy (%)	13	2	2	11	5

p-value ^a< 0.05, ^b<0.01, ^c< 0.001 when compared to the study cohort.

3.5.1 The intra- and inter- rater agreement of calcium scoring using computed tomography

Eighty-two percent (n=27) of subjects had evidence of VC on CT. The mean, SD and range observed for each scoring method for rater 1 [SE] and rater 2 [DD] are shown in Table 3.3. Rater 1 had consistently higher average scores than rater 2 for all 3 scoring methods. The range of Agatston and volume scores were similar for rater 1 and rater 2, but was much wider between raters 1 and 2 for the modified Agatston score due to one subject who was scored much higher by rater 1.

Table 3.3 calcium scores obtained using CT for Rater 1 and Rater 2 (n=33)

Scoring method	Mean (SD)	Range
Rater 1		
Agatston score (AU)	3300.0 (7309.1)	0 to 30763
Modified Agatston score (AU)	59918.8 (113732.5)	0 to 509995.2
Volume score (cm ³)	1.5 (3.3)	0 to 14.1
Rater 2		
Agatston score (AU)	2488.2 (5962.4)	0 to 30670
Modified Agatston score (AU)	41833.4 (77835.4)	0 to 277206.3
Volume score (cm ³)	1.1 (2.7)	0 to 14.1

The intra-rater and inter-rater agreement and test-retest reproducibility of CT assessments at all vascular sites and sub-regions can be found in Table 3.4. It was not possible to obtain results at the carotid arteries as only one subject had one small calcified deposit at this vascular site. Characteristics showed this subject was at the higher end of the age range of the study population at 78 years, however the other study characteristics for this subject were otherwise unremarkable. Intra-rater ICC for total scores were 0.98-0.99 for both raters. Intraclass correlations tended to be highest at the AA and IA, ranging from 0.70 to 0.95 and 0.95 to 0.99 for the AA and iliac's respectively. Inter-rater ICC ranged from 0.43 for the TA to 0.96 for the IA, with the highest ICC observed for total score rather than at individual vascular sites (0.89 to 0.96). The ICC obtained tended to be higher at the AA and IA, with ICC scores of 0.92 for the AA and 0.81 to 0.96 for the iliac's respectively. The TA had the lowest inter-rater ICC (0.43 to 0.49) which failed to reach statistical significance for all three CT scoring methods. Intraclass correlations ranged from 0.97 to 0.98 for test-retest reproducibility obtained by performing duplicate scans. The TA had a slightly lower ICC than the other vascular sites (0.92 to 0.95) while the AA and IA had higher ICC of 0.95 to 0.98 and 0.95 to 0.98 respectively for each of the scoring methods. The ICCs observed were consistent for intra-rater, inter-rater and test-retest agreement regardless of the scoring CT scoring method applied (Table 3.4).

Table 3.4 Intra-rater, inter-rater and test-retest intraclass correlations (and 95% CI) for calcium scoring using CT.

	Rater 1	Rater 2		
	Intra-rater	Intra-rater	Inter-rater	Test-retest
Agatston Score				
Total	0.98 (0.95 - 0.99)^c	0.98 (0.97 - 0.99)^c	0.96 (0.92 - 0.98)^b	0.98 (0.94-0.99)^c
*Carotids	-----	-----	-----	-----
Thoracic aorta	0.83 (0.66 - 0.92) ^c	0.87 (0.74 - 0.93) ^c	0.43 (-0.16 - 0.72)	0.93 (0.63-0.98) ^b
Abdominal aorta	0.86 (0.72 - 0.93) ^c	0.70 (0.39 - 0.85) ^b	0.92 (0.83 - 0.96) ^c	0.95 (0.73-0.98) ^c
Iliacs	0.98 (0.97 - 0.99) ^c	0.99 (0.99 - 0.99) ^c	0.96 (0.91 - 0.98) ^c	0.98 (0.90-0.99) ^c
Modified Agatston score				
Total	0.98 (0.95 - 0.99)^c	0.99 (0.98 - 0.99)^c	0.89 (0.79 - 0.95)^c	0.98 (0.92-0.99)^c
*Carotids	-----	-----	-----	-----
Thoracic aorta	0.63 (0.25 - 0.82) ^b	0.97 (0.95 - 0.98) ^c	0.43 (-0.15 - 0.72)	0.95 (0.74-0.99) ^c
Abdominal aorta	0.95 (0.91 - 0.97) ^c	0.92 (0.84 - 0.96) ^c	0.92 (0.85 - 0.96) ^c	0.98 (0.92-0.99) ^c
Iliacs	0.96 (0.91 - 0.98) ^c	0.99 (0.99 - 0.99) ^c	0.81 (0.62 - 0.91) ^c	0.98 (0.89-0.99) ^c
Volume score				
Total	0.98 (0.95 - 0.99)^c	0.98 (0.97 - 0.99)^c	0.96 (0.92 - 0.98)^c	0.97 (0.86-0.99)^c
*Carotids	-----	-----	-----	-----
Thoracic aorta	0.84 (0.67 - 0.92) ^c	0.88 (0.76 - 0.94) ^c	0.49 (-0.04 - 0.75) ^a	0.92 (0.60-0.98) ^b
Abdominal aorta	0.86 (0.73 - 0.93) ^c	0.71 (0.41 - 0.85) ^c	0.92 (0.84 - 0.96) ^c	0.95 (0.73-0.99) ^c
Iliacs	0.98 (0.97 - 0.99) ^c	0.99 (0.99 - 0.99) ^c	0.96 (0.91 - 0.98) ^c	0.95 (0.78-0.99) ^c

p-value ^a <0.05, ^b <0.01, ^c <0.001. * ICC was not calculated at the carotid arteries as only 1 subject had one small calcification at this vascular site.

Figure 3.5 shows Bland–Altman plots for intra- and inter-rater agreement and test-retest reproducibility for the modified Agatston scoring method. The Bland-Altman plots for each of the CT calcium scoring methods were broadly equivalent therefore only those for the volume scoring method are presented in this chapter. For intra-rater agreement a negative bias was observed for each scoring method indicating that rater 1 scored consistently higher at the first scan reading compared to the second. Figure 3.5A shows a negative bias of -0.320 cm^3 (22%) with three scores that fell outside the limits of agreement (LoA). These subjects had large quantities of calcification within the AA, TA and IA. The inter-rater agreement between raters 1 and 2 for each of the three scoring methods also showed a negative bias indicating that rater 1 (clinical scientist) scored consistently higher than rater 2 (Consultant Radiologist). Figure 3.5B shows inter-rater agreement for the volume score with a negative bias of -0.407 (29%) with one score that fell outside the LoA showing a large difference between rater 1 and rater 2. This subject had large amounts of calcification within both the AA and IA. Results for this subject were also found to be outside the LoA on the Intra-rater Bland-Altman plot. Test-retest reproducibility for scan 1 and scan 2 showed a negative bias indicating that scan 1 was scored higher than scan 2 with a bias of -0.290 and a percentage difference of 5% (Figure 3.5C). Only one score fell outside the LoA. This subject was also found to be outside the LoA on the intra- and inter-rater agreement Bland-Altman plots with calcification in the AA and iliac arteries.

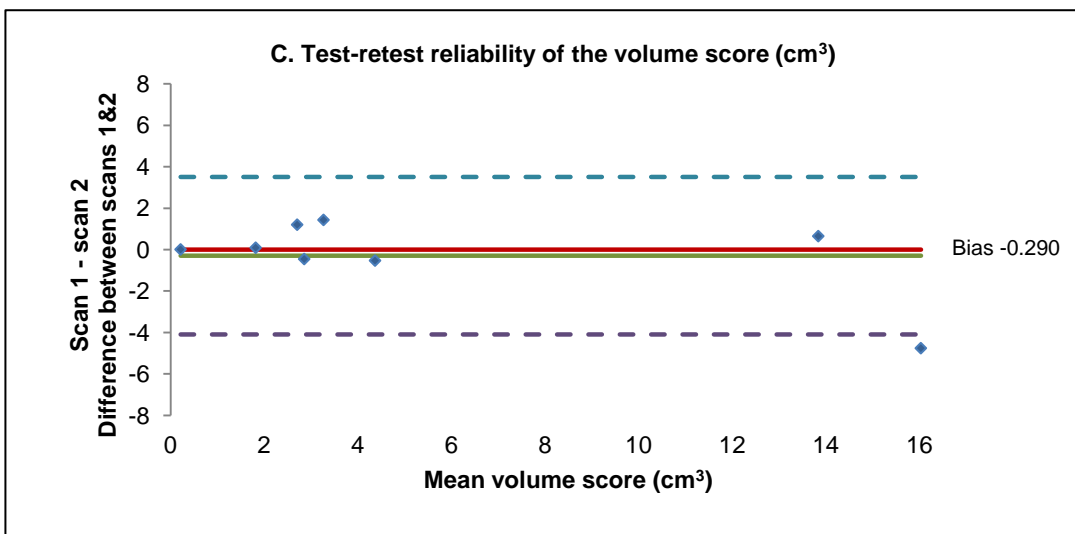
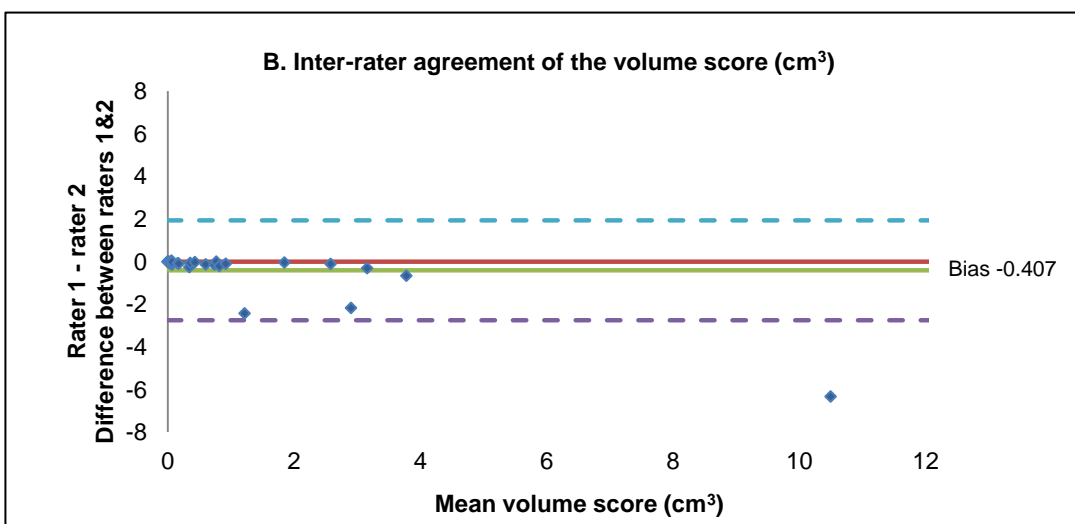
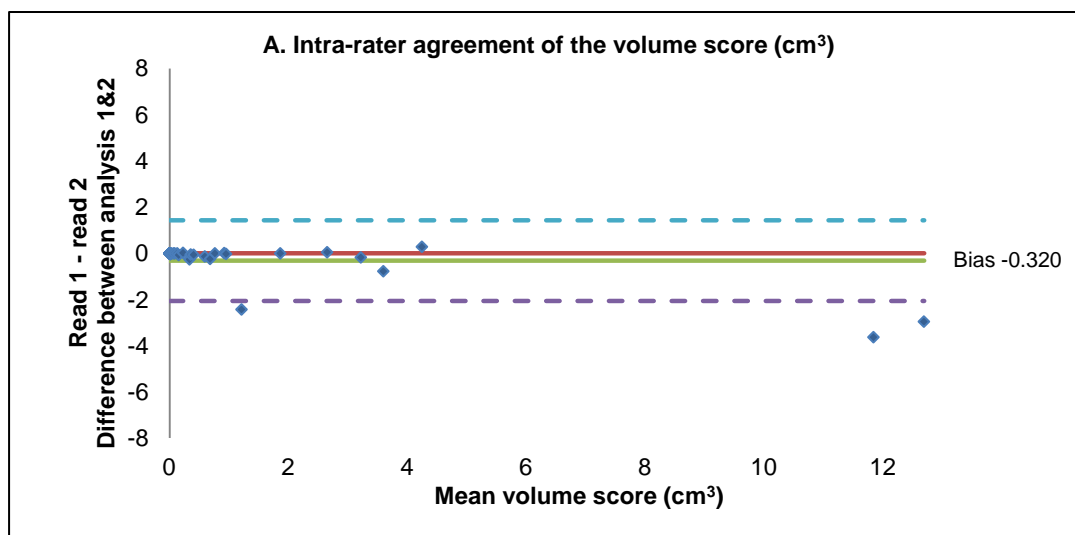


Figure 3.5 Bland-Altman plots are showing (A) intra-rater agreement, (b) inter-rater agreement and (c) test-retest reproducibility (C) for the volume scoring method. The bias line is shown in green with dashed lines showing the upper and lower limits of agreement ± 1.96 SD.

3.5.2 The intra- and inter rater agreement of calcium scoring using lateral VFA

Abdominal aortic calcification was observed on lateral VFA scans in 60% (n=18) of subjects. The mean, SD and range obtained for rater 1 [SE] and 2 [MF] for the 8- and 24-point scoring methods used to quantify calcium using lateral VFA are shown in Table 3.4. Twenty-three percent (n=7) and ten percent (n=3) of the scans were unable to be analysed by raters 1 and 2 respectively as a result poor image quality due primarily to abdominal obesity and/or image artefacts. Figures 3.6 to 3.9 show example lateral VFA scan images acquired in this study. Figure 3.6 is an example of a good-quality lateral VFA image with no evidence of calcification in either the anterior or posterior aortic walls of the AA. Figure 3.7 shows calcification within the anterior wall of the AA at the level of L1-L3 with a small amount of bowel gas at the anterior wall of the AA at the level of L4. Figure 3.8 demonstrates a poor image quality scan taken from a subject with abdominal obesity and Figure 3.9 is an image from a subject with a large amount of bowel gas precluding identification and scoring of calcification within the AA.



Figure 3.6 Example of a lateral VFA scan with no evidence of calcification within the anterior or posterior aortic walls.



Figure 3.7 Example of a lateral VFA scan image with calcification of the anterior aortic wall at L1, L2 and L3 with a 24-point score of 4 and an 8-point score of 2.

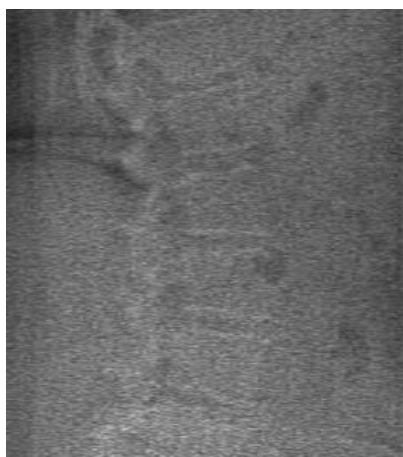


Figure 3.8 Example of a lateral VFA scan with poor image quality due to obesity. This subject had a BMI of 40kg/m²



Figure 3.9 Example of a lateral VFA scan image with profuse bowel gas obstructing the abdominal aortic anatomy.

The scores for the 24-point scoring method did not exceed 11 and 9 for rater 1 and 2 respectively. The 8-point scores did not exceed 5 for raters 1 and 2 (Table 3.5).

Table 3.5 Calcium scores obtained using lateral VFA for rater 1 and rater 2 (n=30)

Scoring method	Mean (SD)	Range	% scans excluded
Rater 1			23%
24-point	2.6 (2.9)	0 to 11	
8-point	1.6 (1.3)	0 to 5	
Rater 2			10%
24-point	2.3 (2.5)	0 to 9	
8-point	1.3 (1.3)	0 to 5	

The Intra-rater, inter-rater agreement and test-retest reproducibility of lateral VFA using the 8- and 24-point scoring methods is shown in Table 3.6. Intra-rater ICC was 0.84 and 0.82 for Rater 1 and 0.93 and 0.93 for Rater 2 for the 24- and 8-point scores respectively. Inter-rater agreement was 0.49 and 0.53 for the total 24- and 8-point scores respectively (not statistically significant). When calcium within the anterior and posterior walls was assessed separately intra-rater and inter-rater agreement was lower on average for the anterior compared with the posterior aortic wall (Table 3.6). Test-retest ICC was 0.94 for the total 24-point scoring method and 0.91 for the total 8-point scoring method.

Table 3.6 Intra-rater, inter-rater and test-retest intraclass correlations (and 95% CI) for lateral VFA.

	Rater 1	Rater 2		
	Intra-rater	Intra-rater	Inter-rater	Test-retest
24-point score				
Total	0.84 (0.62-0.93) ^c	0.93 (0.85-0.97) ^c	0.49 (-0.23-0.78)	0.94 (0.83-0.98) ^c
Anterior aortic wall	0.90 (0.72-0.96) ^c	0.86 (0.71-0.94) ^c	0.64 (0.13-0.85) ^a	0.93 (0.78-0.97) ^c
Posterior aortic wall	0.85 (0.55-0.94) ^c	0.80 (0.56-0.90) ^b	0.48 (-0.25-0.78)	0.93 (0.79-0.97) ^c
8-point score				
Total	0.82 (0.58-0.92) ^c	0.91 (0.81-0.96) ^c	0.53 (-0.13-0.80)	0.91 (0.72-0.97) ^c
Anterior aortic wall	0.86 (0.62-0.95) ^c	0.86 (0.69-0.93) ^c	0.46 (-0.30-0.77)	0.82 (0.46-0.93) ^b
Posterior aortic wall	0.78 (0.40-0.92) ^a	0.67 (0.24-0.85) ^a	0.64 (0.15-0.85) ^a	0.89 (0.69-0.96) ^c

p-value ^a = <0.05, ^b = <0.01, ^c = <0.001

Figure 3.10 shows Bland–Altman plots for the 24-point scoring method. The Bland–Altman plots for the two lateral VFA scoring methods were broadly equivalent and therefore only plots for the 24-point scoring method are shown. For intra-rater agreement a positive bias of 0.6 (26%) was observed for the 24-point scoring method indicating that rater 1 scored consistently higher at the first scan reading compared to the second with only 1 score falling outside the LoA (Figure 3.10A). The study subject whose score fell outside the LoA had bowel gas at L3 and L4 on the scan image which, on further review following statistical analysis, had been misinterpreted as calcification at the first scan read but not the second. The inter-rater agreement between raters 1 and 2 for each of the scoring methods also showed a positive bias of 0.4 (16%) indicating that rater 1 scored consistently higher than rater 2 (Figure 3.10B). Only one score fell outside the LoA which was found to be the same study subject whose score also fell outside the LoA on the intra-rater Bland–Altman plot. Test-retest reliability for scan 1 and scan 2 showed a positive bias of 0.5 (19%) indicating that scores for scan 1 were higher than scan 2. All scores remained within the LoA.

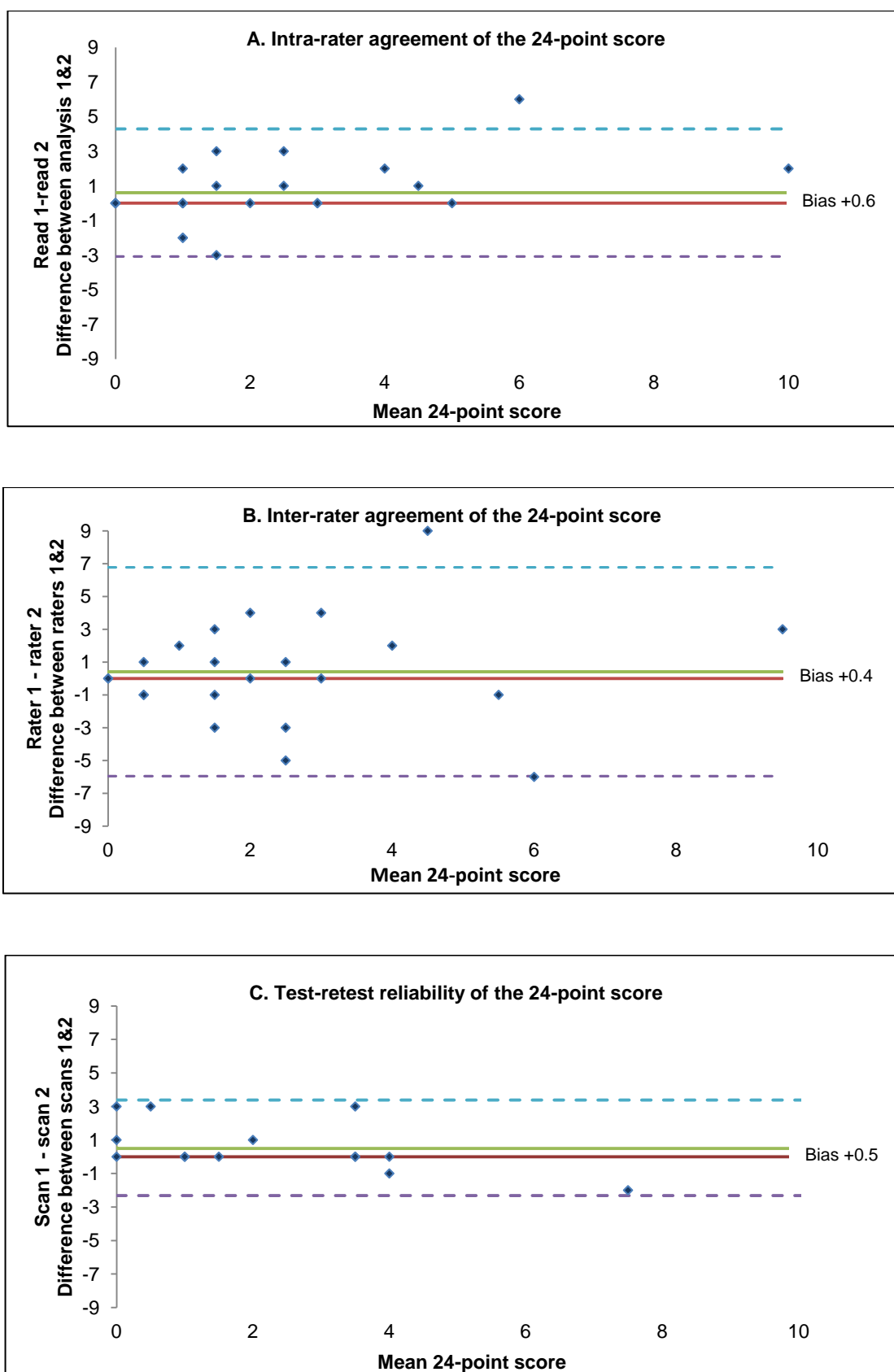


Figure 3.10 Bland-Altman plots showing (A) intra-rater agreement, (B) inter-rater agreement and (C) test-retest reproducibility for the lateral VFA 24-point scoring method. The bias line is shown in green with dashed lines showing the upper and lower limits of agreement ± 1.96 SD.

3.6 Discussion

The rationale for the present study was to investigate the reproducibility and the intra- and inter-rater agreement of lateral VFA scans and moderate dose, non-diagnostic, non-contrast CT for the quantification of VC. There is limited information in the literature on the intra- and inter-rater agreement and precision of CT for quantifying VC at non-coronary sites in low risk populations. The results of this study showed good intra-rater agreement for identifying AAC on lateral VFA scan images. However, results showed the inter-rater agreement of lateral VFA to be relatively poor but not statistically significant. This study showed that intra- and inter-rater agreement for identifying and scoring VC using CT was excellent. Test-retest reproducibility was excellent for both lateral VFA and CT.

In the present study it was difficult to compare results of the intra- and inter-rater agreement of CT with other studies as available data are mainly limited to measurements of coronary calcification. Furthermore, there is also limited information regarding the test-retest reproducibility of CT for quantifying VC at non-coronary sites. However, a small study by Bowden et al in 2009 assessed the interobserver variability between raters for the evaluation of AAC using non-contrast CT scans of the abdomen [568], which demonstrated minimal interobserver variability using the volume scoring method to quantify calcium within a 4.95cm section of the aorta. The present study also reports minimal inter-rater variability. This study included the evaluation of calcification over a larger vascular region, compared with the study by Bowden et al, and included the thoracic and abdominal aorta, as well as the iliac and carotid arteries using three established scoring methods, the Agatston, modified Agatston and volume scores, traditionally used to quantify coronary calcification.

In the present study test-retest reproducibility of CT was assessed to aid in determining if the largest source of error relates to scanner imprecision or intra- or inter-rater variability. The test-retest results in this study were excellent with ICC ranging from 0.92 and 0.98 showing minimal variability between duplicate scans (Table 3.4). In comparison the intra-rater results for CT were more variable with ICC ranging from 0.63 to 0.98 showing that the largest source error was introduced by the rater (Table 3.4). The bias and percentage difference as evaluated using Bland-Altman plots show only minor variation of 5% for test-retest reproducibility (Figure 3.5). Individual calcified deposits on each transaxial CT slice had to first be identified by the rater and then a 'seeding' tool was used to delineate each deposit using the thresholding method described in the methodology i.e. the software did not automatically delineate individual deposits.

Therefore one potential source of variability relates to the possibility that each rater may have overlooked smaller calcified deposit which may not have been visually obvious, or possibly missed due to the large number of slices per subject that had to be analysed. An important point to acknowledge is that it is not possible to remove rater associated errors during image analysis, even during the assessment of test-retest reproducibility of imaging methods, as these scans still require the input of a rater to evaluate the scan images.

Bland-Altman plots presenting intra- and inter-rater agreement (Figures 3.10A & 3.10B) showed there were two subjects that had large variation between scores, between both read 1 and read 2 or between rater 1 and rater 2. Both subjects had a large calcium burden within the AA and iliac arteries. No obvious reason was established which explained the large variance between the scores of the two subjects, although in regions with greater amounts of calcification smaller deposits were often included by rater 1 and not rater 2. Although both raters dedicated a significant amount of time to image analysis in this research study, this may not be possible in a busy clinical environment and highlights a potential issue that could occur in clinical practice.

The ICC values for CT obtained in this study were found to be excellent and in keeping with ICC values reported for other research studies [558, 571]. Acceptable values of ICC that indicate a technique can be used in clinical practice have been discussed in the literature [573, 574], with ICC of 0.75 or greater being considered acceptable for technologies used in health research and clinical practice. The intra- and inter-rater and test-retest ICC in this study for CT measurements at non-coronary sites exceed the recommended minimum showing that the methods used are adequate for this study and for future clinical research.

One of the aims of this part of the study was to evaluate three calcium scoring methods traditionally used to quantify coronary calcification on CT to establish which method would be best for quantifying VC for subsequent adoption in the main clinical study. This study found the Agatston, modified Agatston and volume scores to be broadly equivalent with ICC values between 0.89 - 0.99 with the same outliers present on the Bland-Altman plots for each of the CT scoring methods. Intra- and inter-rater and test-retest ICC were improved by the addition of the IA in the analysis. The Iliacs were initially included to enable the identification of calcified deposits at the aortic bifurcation, often a site of increased deposits, and so preventing the possible exclusion of a scan slice or slices with increased calcification due to differences in the rater's opinion of which transaxial slice includes the bifurcation. The study by Bowden et al, described above, highlighted this as a potential source of error. It was not possible to remove errors associated with

start and end slices entirely and any inconsistency between start points could potentially result in a large variation between scores e.g. due to the inclusion of calcification from the femoral arteries. However, for this study a review of the start and end slice for each vascular site recorded by each rater confirmed consistency for the IA and all other vascular regions.

In this study there was a high prevalence of calcification in the AA and the IA compared to the TA. It is known that VC is more prevalent within the AA compared to the TA [575, 576], with evidence of AAC from the age of 60 and older, which is significantly associated with CVD mortality [555]. A large study by Honkanen et al (CORD) reported that AAC as detected by lumbar radiographs was present in 81% of the patients with CKD with the largest percentage located at L4, indicating that calcification tends to develop in a distal to proximal direction within the aorta [577]. More recently a study by Allison et al [578] demonstrated that in terms of the extent of calcification, the IA showed the strongest association for all-cause mortality, consistent with the well-known association between the severity of peripheral artery disease and both CVD and total mortality [578]. Furthermore the study concluded that higher levels of calcification in different vascular sites are associated not only with CVD mortality but also with non-CVD and total mortality, and that location of the arterial calcification appears to be relevant to the strength of the association with mortality, and CVD risk factors appear to mediate some of the association [578].

Due to the high radiation dose and cost associated with CT, along with limited scanner availability, there is an obvious rationale for evaluating the use of lateral VFA scans for the detection of AAC, especially as they can be easily acquired concurrently with routine bone densitometry with minimal time. The intra-rater agreement of AAC scored using lateral VFA scans in the present study was good and found to be comparable with other studies [549, 558, 569]. However, inter-rater agreement was poorer, non-significant, and not consistent with the findings in other studies [549, 558]. The study by Schousboe et al [549] reported intra-rater and inter-rater ICC values of 0.81 and 0.89 respectively for quantifying AAC on lateral VFA using the 24-point score which were moderately higher than the inter-rater results obtained in the present study. However, the study by Schousboe did have a larger study population than the present study [549]. A study by Toussaint et al [558] evaluated the intra- and inter-rater agreement of lateral DXA-VFA for evaluating AAC in a high risk group of patients with CKD and reported intra- and inter-rater results closely comparable to the results in the present study with ICC values between 0.89 to 0.91 and 0.84 to 0.90 and 0.53 to 0.68 and 0.60 to 0.70 for the 24- and 8-point scores respectively [558]. A further study by Toussaint et al [569] evaluated the

test-retest reproducibility of lateral DXA-VFA scans for quantifying AAC and reported ICC values ranging from 0.91 to 0.93 and 0.82 to 0.87 for the 24- and 8-point scores respectively. Although the present study focused on a lower risk population, the test-retest results in the present study were found to be comparable to those seen in renal dialysis patients, who had greater amounts of VC on average than the healthy postmenopausal women included here [569].

Another study aim was to evaluate the previously validated semi-quantitative 24- and 8-point scores for quantifying AAC on lateral VFA scan images [339, 564] to establish which method would be best for quantifying AAC for subsequent adoption in the main clinical study. This study found the 24- and 8-point scores to be broadly equivalent with intra-rater ICC values of 0.84 and 0.82 for the total 24- and 8-point scores respectively, and inter-rater ICC values of 0.49 and 0.53 for the total 24- and 8-point scores respectively, with the same outliers present on the Bland-Altman plots for both of the lateral VFA scoring methods. Although the adoption of the simplified 8-point scoring method for use in clinical practice would be considered a sensible approach, results for the 24-point scoring method are presented in subsequent chapters, primarily due to the fact that it has been widely applied to evaluate lateral radiographs acquired in osteoporosis trials and has been validated as an independent predictor of vascular morbidity and mortality [339].

Both the intra- and inter-rater ICC were much lower with lateral VFA compared to CT. Lateral VFA scan images are two-dimensional and identification and quantification of AAC can only be achieved by assessing calcification on lateral scans as described by Schousboe et al [564]. Furthermore image resolution can be influenced by patient size [569], and the identification and scoring of calcification is affected by image quality and the presence of image artefacts such as profuse or focal bowel gas overlying the AA on lateral scans. Advantages of single-energy imaging are that the images can be obtained faster. However, single-energy images - used in the present study - are disadvantaged in that the shadows created by soft tissues can obscure visualisation of the vertebrae, especially in areas where the contrast between adjacent soft tissues is considerable. Dual-energy x-ray IVA imaging using the Hologic scanner on the other hand has better resolution than single-energy but the effective radiation dose is higher with a longer scan acquisition time. Furthermore, DXA-VFA images can be degraded to a greater extent by increasing obesity. Computed tomography scan images enable the reader to identify and score calcification through three-dimensional transaxial slices that allow calcified deposits to be identified around the entire circumference of the arterial lumen [579],

without artefacts caused by overlying soft tissue or bone as for two-dimensional imaging methods, such as lateral VFA [564].

The test-retest reproducibility results for lateral VFA in this study were good with ICC ranging from 0.82 and 0.94 showing that scanner imprecision is minimal (Table 3.6). The intra-rater results were more wide-ranging with ICC ranging between 0.71 to 0.84 and 0.67 to 0.93 for raters 1 and 2 respectively (Table 3.6), showing that the largest source of error was introduced by the rater, consistent with results using CT. The scoring of AAC on lateral VFA scan images is subjective and in some cases scans are unable to be assessed. Poor image quality and image artefacts such as profuse bowel gas can mask calcification within the aorta resulting in under or over scoring at best or at worst having scans that cannot be reliably analysed. Obesity, specifically abdominal obesity, significantly affects the quality of lateral VFA scans, therefore hindering the identification of AAC. In the present study 13% (n=8) of the total study cohort were classified as obese using the conventional method of using BMI ≥ 30.0 .

Bland-Altman plots presenting intra- and inter-rater agreement showed there was only one subject that had large variation between the 24- and 8- point scores obtained by rater 1 and rater 2 (Figure 3.10). This subject did have a moderate amount of bowel gas in the region of L2 and L3 which was interpreted as calcification by one of the raters. The percentage difference with intra-rater and inter-rater agreement as evaluated from the Bland-Altman plots was 26% and 16% respectively. These results may reflect the small sample size used in this part of the study - The percentage bias may have been smaller or more comparable with results reported by Schousboe et al if a larger dataset was included. The percentage difference with test-retest reproducibility was higher than expected at 18% but this is also likely to reflect the small sample size included in this part of the study. However, in this study the intra-rater and test-retest ICC for lateral VFA and CT were similar, albeit with more variable with larger 95% CI for lateral VFA compared to CT, indicating that scores obtained with both imaging methods were reproducible. The consistency between the intra-rater ICC indicate that a single rater is able to obtain similar scores with subsequent scan reads with both imaging methods, despite the semi-automated method of image analysis with CT and the subjectivity of scoring AAC on lateral VFA scans. This demonstrates the importance of staff training if these scans are to be evaluated and scored in clinical practice. Although the scoring methods applied are simple, it would be vital that appropriately-qualified staff spend time with an experienced practitioner, for example a Consultant Radiologist as used in this study, to acquire the skills necessary to accurately evaluate and score calcification in the AA.

In the present study a total of seven of the thirty lateral VFA scans were excluded by rater 1 and 3 by rater 2. Scans were excluded if they were unable to be analysed due to profuse bowel gas and/or image artefacts e.g. surgical clips or metal clothing fastenings. This study did not have the issue of the AA being positioned outside of the FoV as this was taken into account during the scan acquisition to include sufficient soft tissue anterior to the lumbar vertebrae. In contrast, a study by Schousboe et al [564] excluded 39 of 174 lateral VFA scans from analysis due to the AA being outside the FoV. The importance of CVD and its link with bone biology is increasingly being researched in the field of osteoporosis and metabolic bone disease and new bone densitometers are being designed to incorporate not only the assessment of OP and fracture risk, but also the assessment of body composition to evaluate metabolic health and AC as a risk factor for CVD. Hologic have recently introduced the 'Horizon' densitometer that produces vertebral images, similar to the Hologic Discovery used in the present study, in high definition using Horizon's high definition instant vertebral assessment (IVA-HD) feature. This feature is also optimised to visualise AAC which will be useful in future research studies [580].

There were limitations in this study. The study sample size was small which may have contributed to the lower inter-rater ICC results with lateral DXA compared with those reported by others [558]. Only eight subjects underwent duplicate CT scans and nineteen subjects duplicate lateral VFAs to evaluate test-retest reproducibility. Different raters were used for the assessment of inter-rater agreement with lateral VFA and CT image analysis. The second rater analysing lateral VFA scan images was a clinical scientist whereas the second rater analysing the CT images was a Consultant Radiologist. It is important to consider that a clinical scientist will have a different skill set to that of a radiologist and the amount of time dedicated to image analysis will differ greatly between the two. This was reflected in the inter-rater ICC results for CT in the present study, particularly at vascular regions with smaller or fewer calcified deposits. For example, the TA had the lowest inter-rater ICC results which suggest a level of disagreement with the amount of calcification included in the analysis. However, intra-rater ICC results with CT were closely comparable for a Consultant Radiologist and a clinical scientist. Inter-rater ICC results with lateral VFA were low and not significant in the present study. These results are a reflection of the subjectivity associated with calcium scoring using lateral VFA scan images.

3.7 Conclusion

The aim of this study was to investigate the test-retest reproducibility and the intra- and inter-rater agreement of lateral VFA scans and CT for the quantification of VC. The results showed good intra-rater agreement for lateral DXA for quantifying AAC and also good test-retest reproducibility. However, the non-significant results for inter-rater agreement demonstrate lateral VFA may not be suitable in clinical practice where multiple raters will be evaluating scan images. This study also showed excellent intra- and inter-rater agreement and test-retest reproducibility for quantifying VC using moderate dose, non-diagnostic CT. The CT results were superior to those obtained using lateral VFA, an anticipated finding, nevertheless it does demonstrate that moderate dose, non-diagnostic, CT offers an alternative to higher dose (diagnostic) CT used in clinical practice for the quantification of VC at multiple vascular sites. An important secondary objective of this part of the study was to determine if any of the scoring methods applied to quantify calcification, for either technique, was superior. This information, along with correlation between calcium scored on lateral VFA images and AAC measured on CT included in Chapter 4, would determine which scoring method was applied in the core clinical study. In terms of the intra- and inter-rater agreement and test-retest reproducibility each of the scoring methods applied to either lateral VFA or CT scans were broadly equivalent.

Chapter 4

The evaluation of lateral VFA and pulse wave velocity for the assessment of AAC by comparison to the gold-standard CT

4.1 Introduction

Atherosclerosis, a major cause of CVD continues to be the leading cause of mortality in postmenopausal women and the identification of those at risk of CVD and subsequent CV events has relied on the assessment of traditional clinical risk factors such as hypertension, obesity, smoking status, family history and diabetes mellitus [581]. Moreover, AAC, a marker of subclinical atherosclerosis, is a predictor of subsequent CV-associated morbidity and mortality [339, 582-584]. The use of CT is recognised as the 'gold-standard' method for the quantitative assessment of calcification with relatively good precision and accuracy [558-562, 585], however, much of the data available has been limited to measurements of coronary calcification [559, 585] and to high risk populations such as those with CKD [558, 559, 561]. Furthermore, the method is limited by high radiation exposure and cost [562, 563]. It has been demonstrated that AAC detected using lateral lumbar radiographs, traditionally used to assess vertebral deformity, is highly predictive of CV-related events and mortality independent of other clinical risk factors [342, 564]. Lateral vertebral fracture assessment imaging has been shown to be a reasonable substitute for standard radiography to detect vertebral deformities, and can be readily performed at the time of bone densitometry with minimal additional time and radiation exposure [586-588]. The AA can be visualised on lateral VFA scan images anterior to the lumbar spine, and the use of lateral VFA has been proposed as a useful imaging tool for the semi-quantitative assessment of AAC [564], with reasonably good sensitivity and specificity when compared to conventional radiographs [570]. Several studies have assessed the accuracy of lateral VFA scans for quantifying AAC by comparison with the gold-standard of CT. For example, Toussaint et al previously reported good sensitivity of lateral VFA for the assessment of AAC with results closely comparable to that of CT, and suggested that lateral DXA scans may allow concurrent assessment of AAC as well as BMD in women referred for routine screening for low BMD and OP [558]. Most of the studies assessing the sensitivity of lateral VFA for quantifying AAC have been limited to high risk populations with a high incidence and extent of VC [558, 561]. However, a recent study by Cecelja et al

assessed the accuracy of lateral DXA-VFA compared to CT in a group of healthy postmenopausal women and reported good sensitivity and specificity of DXA scan images for predicting AAC as detected by CT, supporting the suggestion that lateral DXA-VFA scan images may provide a useful alternative to CT for quantifying AAC [567].

Pulse wave velocity is a simple, non-invasive method of measuring arterial stiffness (Section 2.10, Chapter 2.0) and has been shown to be an independent predictor of CV-related events and mortality [321, 474, 589-593]. Pulse wave velocity is in part dependent on atherosclerotic burden and/or medial calcification, and is widely used in the fields of renal and diabetic research. The predictive importance of PWV was first highlighted in renal patients for whom the incidence of CV events is greatly increased in patients with a high PWV [321, 590]. These findings have been replicated in other study groups including hypertensive, diabetic and elderly subjects where PWV is a predictor of CV morbidity and mortality independent of other more established CV risk factors including hypertension [591, 592]. A large epidemiological study by Cecelja et al reported aortic PWV to be associated with age and hypertension but not with other clinical risk factors associated with atherosclerosis [329]. In addition to these findings in human studies, a study by Farrar et al demonstrated PWV is not associated with early stage atherosclerosis in primates [594]. Moreover, murine models of medial calcification resulted in increased aortic stiffness independently of the development of atherosclerosis [595]. Previous studies demonstrating an association between aortic stiffness and atherosclerosis and aortic stiffness and VC have not distinguished between calcified and non-calcified atheromatous plaques or between calcification in the presence or absence of atherosclerotic plaques [593, 596]. However, a recent Twins UK research study evaluated the association between aortic stiffness measured using PWV with atherosclerotic and calcified plaque, reporting a significant association between PWV and calcification, with PWV increasing with calcific burden, and the study concluded that aortic stiffening relates to AC but not to atherosclerotic plaque burden [571]. Therefore PWV provides another method for estimating AC, and is the rationale for inclusion in this study. The ability of lateral VFA scan images to detect AAC as determined by the 'gold-standard' of CT remains to be fully-established, particularly within lower risk populations with a larger sample size. Moreover, the relationship between PWV and AAC, a surrogate of subclinical atherosclerosis, remains to be established. A summary of previous studies evaluating the accuracy of imaging methods used to measure VC/AAC which was used to compare and correlate with the results from the present study are presented in Table 4.1.

Table 4.1 Overview of previous studies evaluating lateral VFA, CT and PWV for the quantification of AAC/VC and arterial stiffness.

Author (year)	Population (n)	Mean age (yrs)	Imaging method	Calcium scoring method	Outcome measure	Study outcome
Cecelja (2013) ^[567]	GP (105)	64.2	DXA	24-Point	VC	Good sens
Figueiredo(2013) ^[597]	GP(815)	>65	CT	Volume	AAC	AAC
Toussaint (2009) ^[569]	HD (40)	58.5	Rad	24-Point	AAC	>age
			DXA	8-Point	VC	Good sens
			CT	24-Point	AAC	
Avramovski (2013) ^[598]	HD (60)	59.3	DXA	Agatston	AAC	Sig +
	GP (80)	57.5	PWV		AS	assoc
Cecelja (2011) ^[571]	GP (900)		CT	Agatston	VC	Sig +
			PWV		AS	assoc
Toussaint (2008) ^[561]	CKD (48)	64.5	CT	Agatston	VC	No assoc
			PWV		AS	

HD, haemodialysis; CKD, chronic kidney disease; GP, general population; CT, computed tomography; AS, arterial stiffness; Rad, radiographs; Sens, sensitivity; Sig, significant.

4.1.1 Study aim

The aim of this part of the study was to evaluate the accuracy, sensitivity and specificity of lateral VFA scans for the detection of AAC as measured using the ‘gold-standard’ of CT. A further aim of this study was to evaluate the relationship between aortic stiffness measured using PWV with AAC measured using lateral DXA and VC measured using CT.

4.2 Study population

The study population consisted of 444 healthy, ambulatory postmenopausal women with a mean age of 62 years who had attended the Osteoporosis Unit to take part in a large cross-sectional study as previously described in Section 2.4, Chapter 2. Subjects had carotid to femoral PWV ultrasound measurements to assess aortic stiffness, CT scans of the chest and abdomen and lateral DXA scans to quantify AAC (Section 2.9 & 2.11, Chapter 2).

4.3 Materials and methods

4.3.1 Measurement of PWV

Three hundred-sixty two (82%) of the 444 subjects had carotid to femoral PWV performed using the SphygmoCor (AtCor Medical LTD, Australia) PWV system (Section 2.10, Chapter 2). Measurements were made by one clinical scientist (SE), and any readings which did not conform to the internal quality checks provided by the SphygmoCor software were rejected and subsequently repeated. Measurements were made in triplicate, with mean PWV values used for data analysis. The validation of this method and its reproducibility has been previously described by Asmar et al, with intra- and inter-observer repeatability coefficients of 0.935m/s and 0.890m/s respectively [337]. Measurements of PWV were not obtained for 82 (19%) of the total study cohort of 444 subjects, primarily as a result of excessive amounts of adipose tissue at the femoral artery measurement site precluding an accurate recording of the pressure pulse.

4.3.2 Lateral VFA scans

Three hundred forty (77%) of the 444 subjects had lateral single-energy VFA (IVA-HD) scans of the lumbar spine using the Hologic Discovery QDR 4500 densitometer (Hologic, Bedford MA) (Section 2.9, Chapter 2). Scans were obtained using an established local protocol for lateral VFA scan acquisition which was modified to include the lumbar region only using a rotating C-arm which required the subject to lie supine with raised arms (Section 2.9, Chapter 2). One hundred four (31%) of the 340 lateral DXA scans were unevaluable due to poor image quality primarily as a result of abdominal obesity or overlying bowel gas obscuring the walls of the AA.

4.3.3 Semi-quantitative assessment of AAC

Abdominal aortic calcification was assessed on the lateral VFA scan images by a clinical scientist (SE), blinded to subject demographics and BMD results with scans identified only by the subjects study number. Scans were analysed using the semi-quantitative 24-point and 8-point scoring methods as described in Section 2.9.1 and 2.9.2, Chapter 2 in which both the location and the extent of calcification at each lumbar vertebral segment (L1-L4) were evaluated [564].

4.3.4 CT imaging

One hundred thirteen (25%) of the 444 subjects underwent non-contrast; non-diagnostic, un-gated CT scans of the chest and abdomen. The scans were performed using the Philips Precedence 16-slice SPECT/CT helical scanner (Philips Healthcare, Best, Netherlands) as described in Section 2.11, Chapter 2. The scan had a field of view (FoV) of approximately 50cm which included the IA, AA, TA and CA. Scan images were reconstructed into contiguous 3mm transverse slices for viewing on an image analysis workstation. Each scan consisted of 170 slices on average varying with the height of the subject. All scan images were viewed and analysed using the semi-automated hybrid viewer function on Hermes Gold TM imaging software.

4.3.5 Quantitative assessment of AAC on CT

Abdominal aortic calcification was evaluated using CT scans for direct comparison to AAC as measured using lateral VFA scans. Vascular calcification was also assessed at the other vascular sites included during CT scan acquisition. Scans were assessed by one clinical scientist (SE), blinded to subject demographics, BMD results and lateral VFA AAC scores. Scan images were identified only by the subjects' study number. Each 3mm scan slice was analysed individually and calcified deposits were defined as any area greater than 1mm² within the blood vessel being assessed with a HU of ≥ 130 , and lesions were recorded according to anatomical location and deposit number as described in Section 3.3.2, Chapter 3. The total Agatston [349], modified Agatston [553] and volume score [554] were obtained by summing the scores for each cross-sectional slice (Section 2.12, Chapter 2).

4.4 Statistical analysis

Study population characteristics are presented as the mean and standard deviation (SD) unless otherwise stated. The student's t-test and chi-squared test were used to test for significant differences in characteristics between subject groups for PWV and lateral VFA. The Kolmogorov-Smirnov test was used to assess normality of distribution of measures of VC including lateral VFA AAC scores, CT VC scores and PWV. Spearman's correlation coefficient was calculated to assess the correlation between the 24-point and 8-point scores, the correlation between lateral VFA and CT AAC scores and the correlation between PWV and calcium scores obtained using lateral VFA and CT. Following univariate analysis outliers were removed from scatterplots to aid visual interpretation of the results. The sensitivity, specificity, positive predictive value (PPV)

and negative predictive values (NPV) were calculated for the 24-point lateral VFA scoring method for identifying calcium detected by CT with a binary cut-off of 0 for zero calcium and 1 for calcium present. To allow an assessment of whether the accuracy of lateral VFA changes with increasing severity of AAC on CT, sensitivity, specificity, PPV and NPV were calculated for tertiles of CT calcium score for the AA with thresholds set at ≥ 117 , ≥ 670 for the Agatston score, ≥ 2161.9 , ≥ 15304.4 for the modified Agatston score and ≥ 0.074 and ≥ 0.335 for the volume score. Unlike coronary calcium scores which have established cut-points that are associated with increased CV disease incidence; all CT scores at the AA in this study were divided into equal tertiles as no established cut-off points exist for AAC. The area under the curve (AUC) was calculated using receiver operating characteristic (ROC) analysis. Multi-linear regression analysis was used to assess associations between PWV with AAC as measured by lateral VFA and VC measured using CT at multiple vascular sites, correcting for confounders associated with PWV and/or VC. Confounding variables included age, BMI, MAP, anti-hypertension medication and smoking status. P values <0.05 were considered statistically significant.

4.5 Results

Subject characteristics of the total study population are presented in Table 4.2. Subjects had a mean age of 62 years (range 50 to 81) with no prior history of CV events. Fifty-five per cent ($n=244$) of subjects had a family history of CV disease. Thirteen percent ($n=58$) of subjects had confirmed hypertension with 10% ($n=44$) taking antihypertensive medication. Twenty-one per cent ($n=92$) of subjects reported taking prescribed calcium and vitamin D supplements at the time of the investigations.

Table 4.2 Study total population characteristics (n=444)

	Mean (SD)
Age (years)	61.7 (6.4)
Height (m)	1.62 (0.07)
Weight (kg)	65.8 (12.0)
BMI (kg/m ²)	24.9 (4.4)
Total cholesterol (mmol/l)	6.0 (0.9)
Total triglycerides (mmol/l)	1.0 (0.4)
HDL-cholesterol (mmol/l)	2.0 (0.5)
LDL-cholesterol (mmol/l)	3.5 (0.8)
Mean arterial pressure (mm Hg)	93.6 (10.0)
PWV (m/s)	8.9 (1.7)
Lumbar spine T-score	-1.3 (1.2)
Total hip T-score	-0.8 (0.9)
Hip neck T-score	-1.2 (0.9)
Prescribed Ca/vit D supplement user (%)	21
Family history CVD (%)	55
Hypertension (%)	13
Hypertension treatment (%)	10
History of fracture (%)	31
Current Smoker (%)	6

Frequency distributions for AAC on lateral VFA scans quantified using the 24- and 8-point scores are shown in Figure 4.1, and frequency distributions for VC evaluated on CT using the Agatston, modified Agatston and volume scores are shown in Figure 4.2. The distribution of scores for each of the quantitative measures of calcification were positively skewed ($p < 0.001$) with a large proportion of subjects having zero calcification, as shown on the frequency graphs, averaging 40% of the population using VFA and 23% using CT (Figures 4.1 & 4.2).

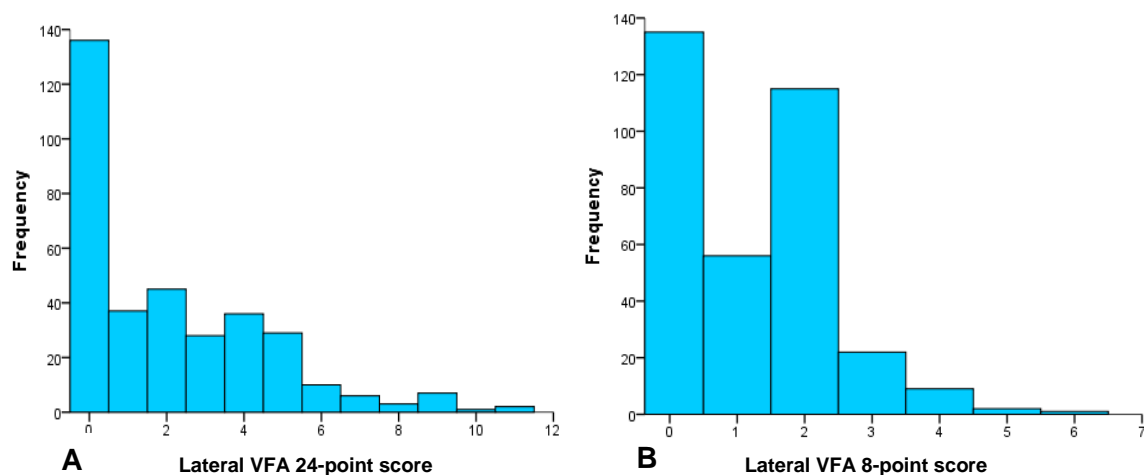


Figure 4.1 Frequency distribution histograms of the 24-point AAC score (A) and the simplified 8-point AAC score (B) from lateral VFA scan images (n=340).

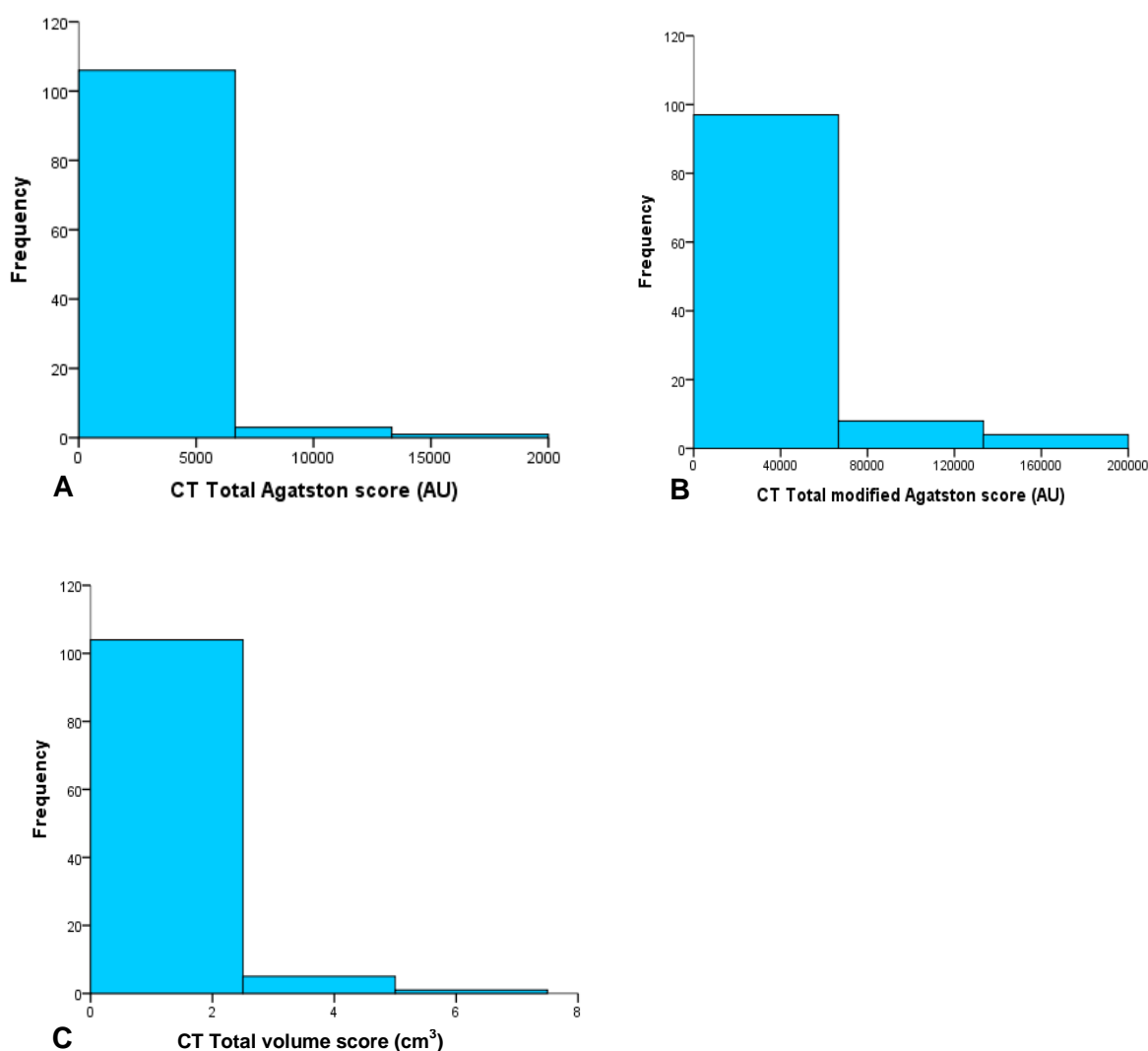


Figure 4.2 Frequency distribution histograms of the total Agatston score (A), total modified Agatston score (B) and total volume score (C) from CT scan images which includes measurements of VC from the IA, CA, AA and TA (n=113). To aid visual interpretation of the distribution of CT scores outliers were removed from each of the histograms.

4.5.1 Relationship between lateral VFA and CT

Calcification was present in 78% of subjects who had CT scans, and 60% of subjects who had lateral VFA scans. Ninety-seven of the 104 unevaluable scans were excluded due to overlying bowel gas and 7 were unevaluable due to obesity affecting the image quality. Statistical differences between subjects with lateral VFA images that could be evaluated and subjects with unevaluable lateral VFA images are presented in Table 4.3. Subjects with unevaluable lateral VFA scan images had a lower body weight and BMI, lower total-cholesterol, triglycerides and LDL-cholesterol, PWV and hip BMD T-scores and higher HDL-cholesterol levels.

Table 4.3 Study characteristics for subjects with lateral VFA scans that were evaluable versus subjects with scans that were unevaluable.

	Evaluable VFA images (n=340)	Unevaluable VFA images (n=105)
	Mean (SD)	Mean (SD)
Age (years)	61.6 (6.3)	61.9 (6.6)
Height (m)	1.62 (0.7)	1.63 (0.0)
Weight (kg)	67.5 (11.5)	60.0 (12.0) ^c
BMI (kg/m ²)	25.6 (4.3)	22.6 (4.2) ^c
Total cholesterol (mmol/l)	6.0 (0.9)	5.9 (0.9)
Total triglycerides (mmol/l)	1.0 (0.5)	0.9 (0.3) ^a
HDL-cholesterol (mmol/l)	2.0 (0.5)	2.2 (0.5) ^c
LDL-cholesterol (mmol/l)	3.6 (0.8)	3.3 (0.8) ^c
Mean arterial pressure (mm Hg)	93.8 (10.1)	93.0 (9.8)
PWV (m/s)	9.0 (1.8)	8.4 (1.5) ^b
Lumbar spine T-score	-1.3 (1.2)	-1.4 (1.2)
Total hip T-score	-0.7 (0.9)	-1.0 (0.9) ^b
Femoral neck T-score	-1.1 (0.9)	-1.4 (0.9) ^c

p-value ^a<0.05, ^b<0.01, ^c<0.001 when compared to subjects with lateral VFA scans that could be evaluated.

The mean total Agatston, total modified Agatston and total volume scores for VC measured using CT were 2442.6 ±10129.9 arbitrary units (AU), 39787.4 ±115565.8 AUs and 1.15 ±4.57cm³ respectively (Table 4.4). The mean total 24- and total 8-point scores for AAC as measured using lateral VFA images were 2.12 ±2.42 and 1.19 ± 1.16 respectively (Table 4.4). Mean VC scores were calculated for each vascular site using CT. The AA and IA had the highest extent of calcification while the lowest incidence and extent of calcification was observed in the CA (Table 4.4).

Table 4.4 Calcium scores for individual vascular sites and total scores obtained using CT and lateral VFA.

Scoring method	Mean (SD)	Range
CT Agatston (AU)		
Total	2442.6 (10129.9)	0-98782
Carotid arteries	18.3 (108.0)	0-1026
Thoracic aorta	202.19 (952.2)	0-9355
Abdominal aorta	1294.9 (5548.6)	0-55618
Iliac arteries	927.2 (3910.2)	0-33733
CT Modified Agatston (AU)		
Total	39787.4 (115565.8)	0 - 1019091.2
Carotid arteries	447.1 (3000.0)	0 - 29291.9
Thoracic aorta	3047.9 (11346.7)	0 - 99876.5
Abdominal aorta	20727.2 (62411.7)	0 - 551780.7
Iliac arteries	15565.1 (50491.4)	0 - 366927.8
CT Volume score (cm³)		
Total	1.151 (4.573)	0 - 44.323
Carotid arteries	0.220 (0.990)	0 - 0.490
Thoracic aorta	0.776 (1.895)	0 - 4.260
Abdominal aorta	1.752 (3.397)	0 - 24.860
Iliac arteries	1.736 (2.930)	0 - 15.155
VFA 24-point score		
Total	2.12 (2.42)	0 - 11
VFA 8-point score		
Total	1.19 (1.16)	0 - 6

Univariate analysis revealed significant positive correlations between each of the scoring methods used to quantify VC on CT. A significant positive correlation was also observed between the semi-quantitative 24-point score and the simplified 8-point scoring method $r = 0.95$, $p = <0.001$. A scatterplot showing the correlation between the 24-point and 8-point AAC scores is presented in Figure 4.3.

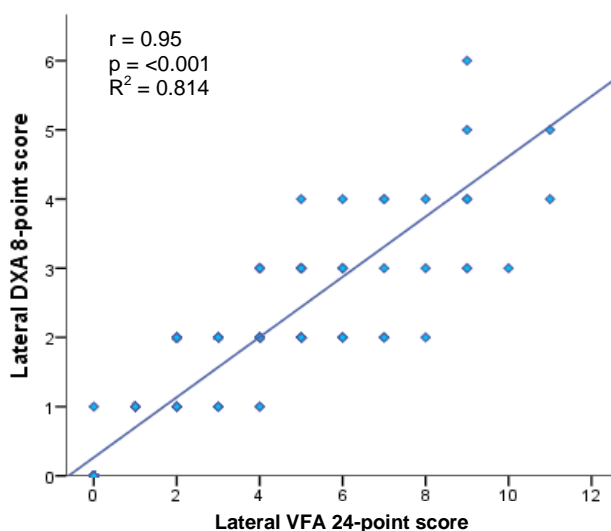


Figure 4.3 Scatterplot of the semi-quantitative 24-point score versus the simplified 8-point score from lateral VFA scans.

Univariate analysis revealed significant positive associations between measures of AAC on lateral VFA scans with measures of AAC on CT scans (Table 4.5). The strength of association was broadly equivalent between measures of AAC assessed on lateral VFA with AAC assessed on CT scans with r -values averaging 0.28.

Table 4.5 Correlations between lateral VFA and CT VFA-matched* AAC scores.

	<i>r</i>	<i>p</i>
24-point score		
Agatston	0.29	0.006
Modified Agatston	0.29	0.005
Volume	0.29	0.006
8-point score		
Agatston	0.27	0.011
Modified Agatston	0.27	0.009
Volume	0.27	0.010

*AAC was measured within the AA adjacent to L1-L4 on CT images as with VFA scans.

Table 4.6 shows the sensitivity, specificity, PPV, NPV and AUC of lateral VFA for detecting AAC measured using CT as the gold-standard. The presence of AAC was correctly identified in 33 (36%) subjects, with false negatives in 27 (30%) subjects who did have AAC (sensitivity 55%). Fifteen subjects were correctly identified as having no

AAC with false positives identified in 16 subjects who did not have AAC (specificity 48%). The probability of subjects identified as having AAC as detected by lateral VFA scans that had AAC as measured by CT (PPV) was 67%, and the probability of subjects identified as having no AAC that were negative for AAC on CT (NPV) was 36%. The area under curve was 0.52 for AAC.

To investigate if the accuracy of lateral VFA changes with different degrees of AAC as detected by CT, the sensitivity, specificity, PPV, NPV and AUC were calculated for tertiles of calcification as measured using CT (Table 4.6). Thresholds were set at each tertile of the variable for the CT AAC scores resulting in the same values for sensitivity, specificity, PPV, NPV and AUC for each of the CT scores. Most of the measures with lateral VFA improved with increasing severity of AAC with moderately-good sensitivity (64%, 83%), specificity (55%, 56%) and area under the curve (60% and 69%). Positive predictive values decreased from 67% to 39% while NPV increased from 36% to 90% with increasing severity of AAC.

Table 4.6. Sensitivity, specificity, NPV, PPV and AUC associated with lateral VFA predicting AAC as detected by CT.

CT AAC Score tertiles*	Sensitivity %	Specificity %	PPV %	NPV %	AUC (95%CI)
Binary cut-off >0	55	48	67	36	0.52 (0.38-0.62)
Tertile 1	64	55	55	64	0.60 (0.47-0.70)
Tertile 2	83	56	39	90	0.69 (0.55-0.80)

*Abdominal aortic calcification as measured using CT was categorised as a binary cut-off point of 0 for no calcium and 1 for calcium present, tertile 1 was categorised as scores ≥ 117 AU for Agatston, ≥ 2161.9 AU for modified Agatston and ≥ 0.074 cm³ for volume scores respectively and tertile 2 as scores ≥ 670 AU for Agatston, ≥ 15304.4 AU for modified Agatston and ≥ 0.335 cm³ for volume scores respectively.

Figure 4.4 shows receiver operating characteristic (ROC) curves for lateral VFA detecting AAC as measured by CT for the lowest to highest tertiles of AAC. The sensitivity and AUC increase with each tertile of AAC as presented in Table 4.6. The AUC at tertile 2 was found to be statistically significant from AUC at tertile 1 and the binary cut-off ($p=0.001$).

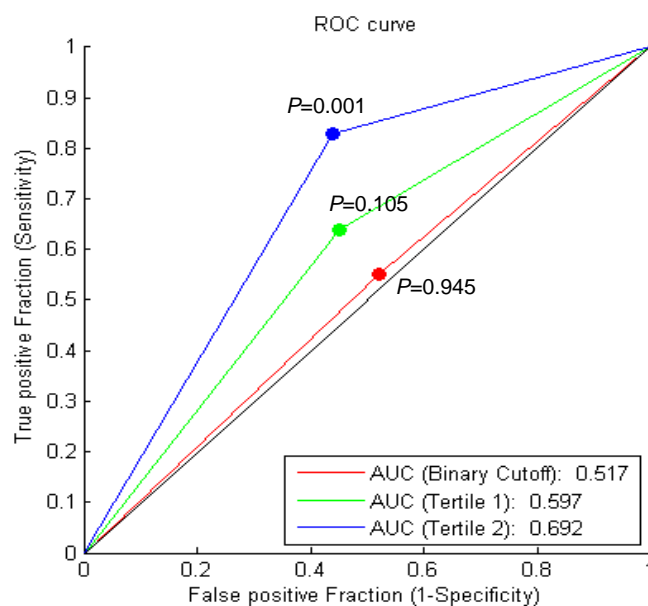


Figure 4.4 ROC curves for lateral VFA detecting the presence of AAC as measured using CT scans with the red, green and blue lines corresponding to tertiles increasing with increased severity of AAC. The red line shows the increase in sensitivity and AUC for AAC set at a threshold of >0 ($p=0.945$), the green line at a threshold of ≥ 117 (AU), ≥ 2161.9 (AU), 0.074 (cm^3), ($p=0.105$); and the blue line at a threshold of ≥ 670 (AU), ≥ 15304.4 (AU), ≥ 0.335 (cm^3) ($p=0.001$) for the Agatston, modified Agatston and volume CT scores respectively.

4.5.2 Relationship between vascular stiffness (PWV) and vascular calcification

Three hundred sixty two (82%) of the total 444 subjects had carotid to femoral PWV performed with a mean PWV result of 8.8 ± 1.7 . The frequency distributions for PWV scores obtained from 362 subjects are shown in Figure 4.5. The distribution of scores was positively skewed ($p < 0.001$) with a large proportion of subjects with a low PWV score, as shown in Figure 4.5.

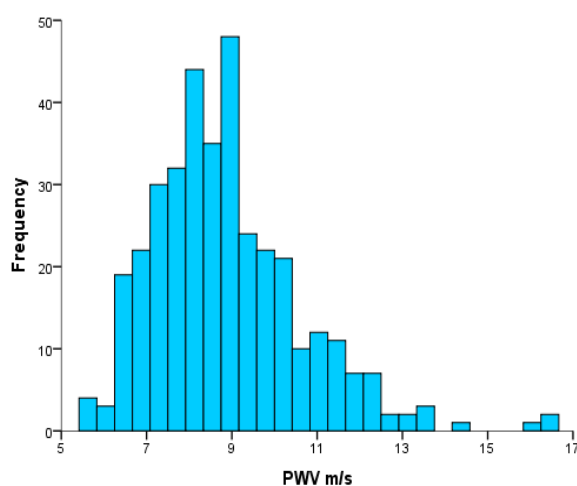


Figure 4.5 Frequency distribution histogram for PWV (n=362).

Statistical differences were observed between subjects that had PWV performed compared with subjects that did not (Table 4.7). Subjects who did not have PWV readings weighed significantly more and had a higher BMI than those that had successful PWV measurements, and subjects without PWV readings had a higher MAP and a lower total-cholesterol than those with PWV readings.

Table 4.7 Study population characteristics for subjects with and without successful PWV measurements.

	Subjects with PWV (n=362)	Subjects without PWV (n=82)
	Mean (SD)	Mean (SD)
Age (years)	61.8 (6.4)	61.3 (6.5)
Height (m)	1.62 (0.07)	1.62 (0.08)
Weight (kg)	64.5 (9.9)	71.4 (17.1) ^b
BMI (kg/m ²)	24.4 (3.5)	27.1 (6.7) ^b
Total cholesterol (mmol/l)	6.1 (0.9)	5.8 (0.8) ^b
Total triglycerides (mmol/l)	1.0 (0.5)	1.0 (0.4)
HDL-cholesterol (mmol/l)	2.0 (0.5)	1.9 (0.4)
LDL-cholesterol (mmol/l)	3.6 (0.8)	3.3 (0.8)
Mean arterial pressure (mm Hg)	93.0 (9.3)	96.4 (12.8) ^a
Lumbar spine T-score	-1.4 (1.2)	-1.2 (1.3)
Total hip T-score	-0.8 (0.9)	-0.6 (1.1)
Femoral neck T-score	-1.2 (0.9)	-1.1 (1.0)

p-value ^a < 0.05, ^b < 0.01 when compared to subjects who had PWV performed.

There were no significant correlations observed between PWV and AAC measured using lateral VFA in univariate analysis (Table 4.8).

Table 4.8 Correlation between PWV and AAC measured using lateral VFA

VFA AAC scoring method	<i>r</i>	<i>p</i>
24-point	0.03	0.570
8-point	0.02	0.687

In contrast, significant positive correlations were obtained when PWV was compared to VC measured using CT (Table 4.9). Figure 4.6 shows scatterplots of the correlations between PWV and the total Agatston, total modified Agatston and total volume VC scores following the removal of outliers. Two outliers were excluded from the Agatston scores, and one outlier from the modified Agatston and volume scores. There was minimal difference in the associations between PWV and VC with results changing from $r = 0.34$, $p = 0.001$; $r = 0.39$, $p = <0.000$ and $r = 0.39$, $p = <0.000$ for the Agatston, modified Agatston and volume scores respectively.

Table 4.9 Correlation between PWV and VC measured using CT.

CT VC scoring method	<i>r</i>	<i>p</i>
Total Agatston	0.39	<0.001
Total modified Agatston	0.40	<0.001
Total volume	0.39	<0.001

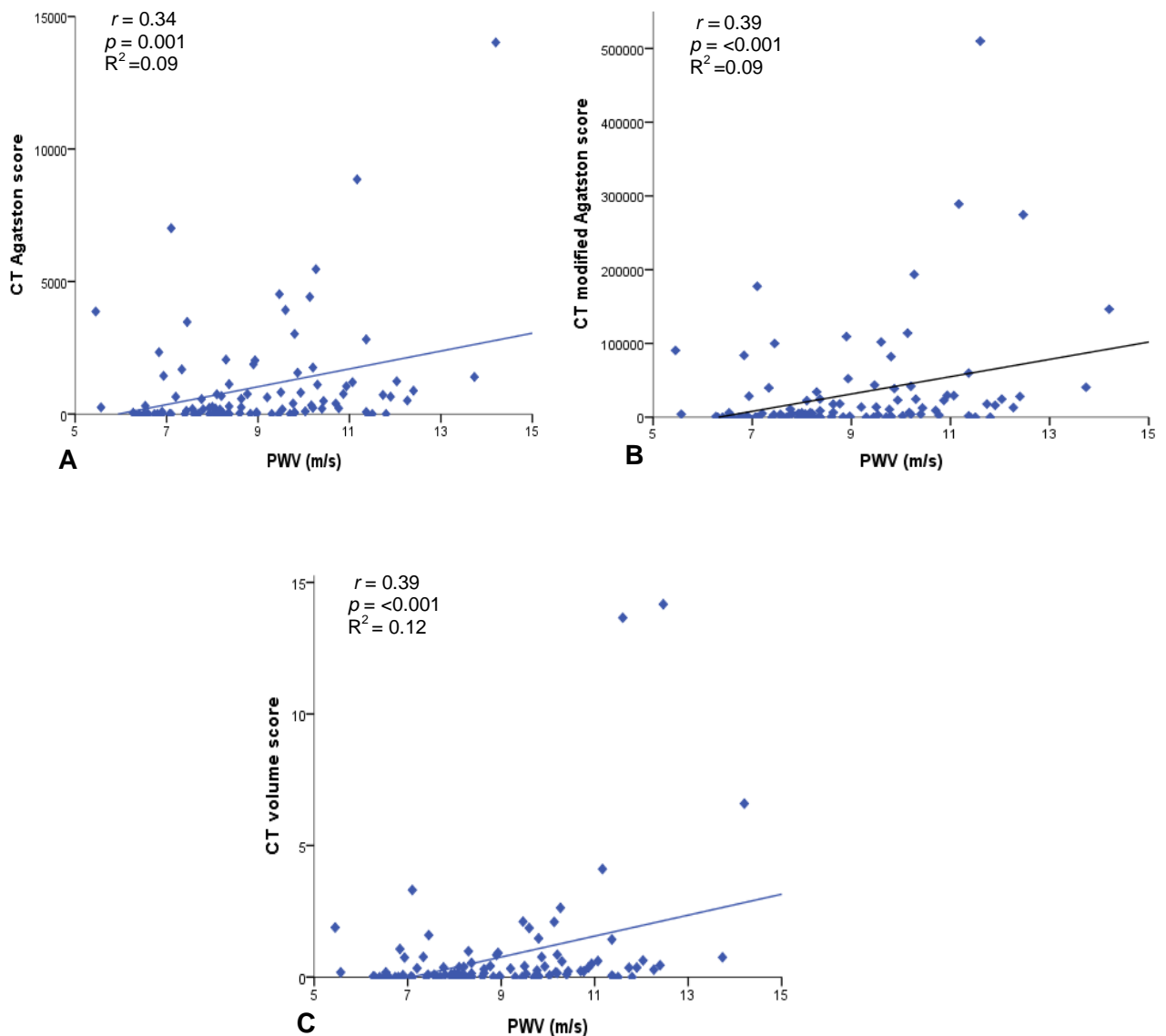


Figure 4.6 Scatterplots showing correlations between PWV and VC quantified using the CT total Agatston (A), total modified Agatston (B) and volume scores (C) following the exclusion of outliers from the scatterplots to aid interpretation of the results.

There were no independent associations between PWV and VC measured using the CT Agatston, modified Agatston and volume scores following adjustment for confounders associated with both arterial stiffness and VC including age, BMI, MAP and hypertension medication and smoking status (Table 4.10). Spearman's correlation showed the three CT calcium scores to be highly correlated, and were therefore included separately into the regression model to avoid collinearity. Age, BMI, MAP and the use of hypertension medication were all found to be significantly positively correlated with PWV, with age being the largest contributor to the regression model.

Table 4.10 Multi-linear regression analysis of arterial stiffness measured by PWV as the dependent variable and the total Agatston, total modified Agatston and total volume VC scores as an independent variables.

Variables	R ²	β-coefficients	p-value
Agatston VC score (AU)	0.40	0.051	0.537
Age (years)		0.386	<0.001
BMI (kg/m ²)		0.195	0.024
MAP (mmHg)		0.203	0.019
Hypertension medication		0.170	0.043
Modified Agatston VC score (AU)	0.39	-0.001	0.982
Age (years)		0.404	<0.001
BMI (kg/m ²)		0.199	0.023
MAP (mmHg)		0.203	0.019
Hypertension medication		0.171	0.044
Volume VC score (cm³)	0.40	0.053	0.528
Age (years)		0.386	<0.001
BMI (kg/m ²)		0.195	0.024
MAP (mmHg)		0.203	0.019
Hypertension medication		0.170	0.043

Variables included in the model: Age, BMI, MAP, hypertension medication and smoking status.

4.6 Discussion

The rationale for the present study was to investigate the accuracy of lateral VFA for detecting AAC as measured using the 'gold-standard' of CT and to determine if lateral VFA scans can be utilised as a surrogate marker of calcification, a risk factor for CV morbidity and mortality, in postmenopausal women referred for OP screening. Computed tomography is associated with radiation exposure and cost, however lateral VFA scans have been proposed as a reasonable substitute for detecting AAC which can be performed in minimal time with low radiation exposure and cost. Furthermore, the present study examined the associations between PWV - a measure of aortic stiffness - and VC measured using lateral VFA and CT scans to establish how measures of VC correlate with aortic stiffness, which itself has been shown to be a strong independent predictor of CV mortality. The results of this study show moderately-good sensitivity of lateral VFA for predicting AAC as measured by CT, furthermore, the present study reports a significant positive association between PWV and VC as measured using CT.

Vascular calcification is an increasingly important clinical concern, particularly in populations with CKD, type 2 diabetes mellitus and atherosclerosis. The pathophysiology of VC is characterised by the deposition of calcium in the intima and/or media of the arterial wall, resulting in increased vascular stiffness. Most individuals aged 60 and over have calcification to some extent in their major arteries [578] leading to the reduction of arterial elasticity which impairs CV haemodynamics, resulting in morbidity and mortality [491, 556, 557] in the form of hypertension, aortic stenosis, ventricular hypertrophy, myocardial and lower-limb ischaemia, congestive heart failure, and compromised structural integrity [599-601]. Various diagnostic methods are currently used to assess VC, however there is an increased preference for simple, reliable, low radiation dose methods that can be readily implemented into routine clinical practice, such as lateral VFA scans for the semi-quantitative assessment of AAC, which can be performed concurrently with routine bone density screening. The presence of AAC is a marker of both subclinical atherosclerotic disease and arteriosclerosis, and is also an independent predictor of CV morbidity and mortality [339, 602]. The use of lateral VFA for the assessment of AAC during screening for OP may be beneficial as a tool to identify asymptomatic patients at low to intermediate risk of a CV event, particularly the elderly, who may benefit from risk factor modification and further CV screening.

Although subjects included in the present study were healthy postmenopausal women from a lower risk population, a large proportion of the cohort had VC, with 78% of subjects having VC detected by CT imaging and 60% having AAC as detected using lateral VFA imaging. These findings are consistent with those previously reported [567, 597]. A recent study by Figueiredo et al reported radiograph-detected AAC was present in over 60% of a low risk community-dwelling population [597]. It is now accepted that from the sixth decade onwards, most individuals will have evidence of calcification within the major arteries [578, 603]. The population in the present study had a mean age of 62 years which is similar to those reported in other studies of low risk populations [567]; supporting the suggestion that AC is prevalent in later life independent of known CV risk factors [558]. Subjects in the present study had a high extent of calcification within the AA and IA which is consistent with the findings reported in Chapter 3. A recent study by Allison et al [578] also reported a high prevalence of calcification within the AA and IA in a study cohort of over 4500 subjects with a mean age of 56 years. The same study also confirmed that IA calcification showed the strongest association for all-cause mortality, consistent with the well-known association between the severity of peripheral artery disease and both CVD and total mortality [578].

The present study assessed the association between the semi-quantitative 24- and 8-point scoring methods for quantifying AAC on lateral VFA scan images. The Framingham study [560] previously reported the 24-point score to be a significant predictor of CV events and mortality independent of other known clinical risk factors. However, a more simplified 8-point score has been proposed as a quick method of assessing AAC on lateral VFA scans, which may be more suitable for use in clinical practice. Ultimately, a quick and simple scoring method for quantifying AAC for the assessment of future CV risk may be adopted into clinical practice for women referred for routine OP screening, which may be useful for identifying patients that can benefit from further treatments such as lipid lowering or anti-hypertensive therapy to reduce the risk of future CV related events. Results of the present study found the 24- and 8-point scores to be highly correlated ($r=0.96$ $p<0.001$), a finding consistent with other studies [18,20], [558, 561, 570]. The strong correlation between the two lateral VFA scoring methods suggests the simplified 8-point score offers a quick tool for the assessment of AAC as an alternative to the widely used and validated 24-point scoring method.

The present study found moderate agreement between AAC measured using lateral VFA scans and VFA L1-L4 matched AAC scores using CT (Table 4.5). Although statistically significant, the correlations were weak accounting for less than 10% of the variance. The strength of association was broadly equivalent between the 24- and 8-point scores and each of the CT VFA-matched AAC scores. The correlations observed in the present study were lower than those of other studies, for example Cecelja et al reported a correlation of $r = 0.58$ between the DXA 24-point score and CT volume score [567]. The correlation between lateral VFA and CT is expected to be higher in subjects with greater amounts of calcification due to a larger range of values observed. For example, Cecelja et al measured AC over a larger vascular area, between the aortic arch and aortic bifurcation on CT [567], compared with the present study which measured AAC between L1-L4 on CT. The semi-quantification of AAC on lateral VFA images is highly subjective. With single-energy VFA, overlying bowel gas and greater quantities of soft tissue as a result of obesity can greatly affect image quality, precluding adequate image analysis which may result in inaccuracies during scoring of AAC on lateral VFA scan images, which would adversely influence the association between AAC measured using VFA and VC measured using CT. Finally, it is impossible to state whether the moderate correlation seen between AAC measured using VFA and AAC measured using CT in the present study represents atherosclerotic burden or arteriosclerosis. Intimal or atherosclerotic calcification is characteristically patchy whereas arteriosclerotic or medial calcification is characteristically more diffuse in

appearance but since the medial and intimal layers of the arterial lumen are in close proximity, it is impossible to distinguish between the two on either lateral VFA or CT images.

There is limited information in the literature reporting the accuracy of lateral VFA for the prediction of VC using CT in low risk populations. The information available reports data on high risk populations with renal disease that tend to have a greater incidence and extent of VC [558, 569]. For example, Toussaint et al evaluated the use of lateral DXA-VFA scans for determining AAC as measured by CT in a cohort of 40 CKD-5 patients and reported that lateral DXA imaging can accurately detect AAC with good sensitivity and specificity [569]. However, recent research from the Twins UK study group evaluated the accuracy of lateral DXA-VFA in a group of postmenopausal women reporting reasonably good accuracy which improved with increasing calcium burden [567].

The present study reported moderately-good sensitivity with the lateral VFA 24-point score for predicting AAC as detected by the 'gold-standard' of CT, which is consistent with results reported by others [567]. Moreover, the accuracy of lateral VFA imaging for predicting AAC on CT improved with the presence of a higher degree of AAC as detected on CT, which is consistent with the results from studies on high risk CKD and dialysis patients [567, 569]. For example, Cecelja et al reported a sensitivity of 56% for correctly detecting the presence of calcification ($AAC > 0$) using lateral DXA-VFA compared to CT in a healthy low risk population, with an increase in sensitivity when analysis was limited to the upper tertiles of the CT calcium scores. Toussaint et al reported a higher sensitivity of 72% which again increased with the severity of AAC [569]. Chronic kidney disease accelerates the development of atherosclerosis and it has been demonstrated that CKD causes excessive vascular inflammation and calcification [604] and therefore the higher sensitivity reported by Toussaint is likely to be a reflection of the greater extent of intimal calcification detected on lateral VFA scans in patients with CKD.

In the present study, the specificity of lateral VFA was much lower than that reported by others [567, 569], with other studies reporting a higher specificity of 80% [567] and 75% [569]. Due to the subjective nature of calcium scoring on lateral VFA scan images, a proportion of scans were incorrectly identified as having calcification when calcium was not in fact present on CT (i.e. false positives). These results also reflect the subjectivity of calcium scoring on single-energy VFA scan images compared with dual-energy VFA scan images that are not disadvantaged by shadows and artefacts created by soft

tissue. The AUC results from this study increased from 0.52 to 0.69 with increasing vascular burden on CT but only reached statistical significance at the highest tertile (Figure 4.4), and therefore indicate a moderate discriminatory value of lateral VFA in the presence of greater quantities of AAC. These ROC results are comparable to those reported by Toussaint et al and Cecelja et al of 0.63 to 0.76 and 0.68 to 0.67 respectively [567, 569]. The negative and positive predictive values in the present study reflected the prevalence of AAC within this study population of lower risk postmenopausal women. Toussaint et al reported higher NPV of 76.9 and 92.3% for the upper tertiles of AAC respectively [569]. In the present study PPV decreased with each tertile of AAC with fewer subjects having high AAC scores. In contrast, the NPV increased which was a reflection of the ability of lateral VFA to accurately predict when subjects did not have AAC. Given the importance of sensitivity, specificity, PPV and NPV, and that they are a critical measure of the performance of lateral VFA for detecting AAC as measured on CT, the moderate scores obtained in the present study would not be adequate for detecting and quantifying low levels of AAC and demonstrate that lateral VFA may only provide an adequate substitute for CT for the detection of high levels of AAC in clinical practice, with values of 70% and higher considered acceptable. The sensitivity and NPV in the present study exceeded 70% only at the highest tertile where the greatest amounts of AAC were measured (Table 4.6). This perhaps should not be considered a negative finding since the use of lateral VFA to identify only those with more severe VC would seem a sensible approach to adopt in clinical practice due to the high incidence of AC with ageing. Reporting the results for only those with the greatest extent of calcification would limit referrals for further investigations to only those patients who may be at greatest risk of a CV event, and importantly avoid unnecessary concern to the patient.

Arterial stiffness as measured by PWV shows little or often no relation to conventional risk factors other than age and blood pressure [329]. Once considered to be largely dependent on atherosclerotic burden, arterial stiffness has been shown more recently to be related to calcified plaque and not non-calcified atheroma [329]. A study by Cecelja et al in 2009 examined the relationship of arterial stiffness to atherosclerosis and measures of VC in a cohort of 900 postmenopausal women and reported arterial stiffening to be correlated with calcified plaques but not with intima-medial thickness or non-calcified atheromas [329]. The study also reported AC as determined by CT was higher in subjects with a high PWV [329]. Animal models of atherosclerosis have also suggested that arterial stiffness is not affected by the early stages of atherosclerosis [594].

The present study found no association between arterial stiffness measured using PWV and AAC as measured using lateral VFA scans (Table 4.8). These results were surprising considering the high incidence of AAC and increased arterial stiffness reported in this cohort of postmenopausal women, and that both PWV and VC increase with age with PWV primarily dependent on calcified plaque burden. In contrast, a recent study by Avramovski et al reported a strong association between PWV and AAC as measured by lateral lumbar radiographs in both normal subjects from the general population and in chronic haemodialysis patients ($p < 0.001$) [598]. The lack of association between PWV and AAC measured using lateral VFA in the present study may be explained by the subjectivity of quantifying AAC on single-energy VFA scan images as already highlighted. Furthermore, because VFA imaging, like most imaging methods, is unable to distinguish between intimal and medial calcification, AAC quantified on lateral VFA images may reflect calcified atherosclerotic plaque burden in contrast to medial calcification which is more strongly associated with arterial stiffness.

In the present study PWV was found to be positively associated with VC as measured using CT with the correlations accounting for less than 16% of the variance (Table 4.9), but this association failed to remain significant after correcting for confounding factors including age, MAP and the use of anti-hypertension therapy which are widely accepted to be significantly associated with arterial stiffness [605, 606]. A significant positive association was observed between PWV and total calcified plaque as measured by CT in a recent twins UK study [571]. The subject demographics in the present study population and those of the twins UK cohort are comparable to the general population of women in the UK for disease and lifestyle characteristics including hypertension and the use of antihypertensive drugs. Toussaint et al also evaluated the associations between VC and arterial stiffness in a study population with CKD and reported a positive correlation between VC and PWV ($r = 0.33$, $p = 0.03$) but like the results from the present study, the association failed to remain statistically significant in multivariate regression analysis [561].

This study did have limitations. The study was limited to lower risk, postmenopausal women and subjects who were known to be taking lipid lowering therapy were excluded, likely lowering both the incidence and extent of VC observed. The study used only moderate dose CT for image acquisition, unlike diagnostic CT scans used in clinical practice that require a higher radiation dose. In addition, it is possible that both lateral VFA and CT imaging differ in their sensitivity to detect intimal and medial calcification. The present study did not address the issue of how well lateral VFA predicts future CV related events and mortality and how those results compare to those using CT.

4.7 Conclusion

The results of this study report moderately-good accuracy of lateral VFA scans for predicting high levels of AAC detected by CT. The present study did not find any associations between PWV and VC. These results demonstrate that lateral VFA scans can provide a low radiation, low cost alternative to the 'gold-standard' of CT for evaluating AAC in a population of postmenopausal women referred for bone density screening for OP who are at a high risk for future CV-related events. Previous studies have evaluated the accuracy of lateral DXA-VFA compared to the 'gold-standard' of CT in both high and lower risk populations, however it is understood this is the first study to assess the validity of lateral VFA for detecting AAC compared to CT with directly comparable regions of interest within the aorta. Further clinical studies are required to determine how well AAC measured by lateral VFA imaging correlates with aortic stiffness as results may ultimately indicate whether PWV is a reliable measure of AC that can be implemented into routine clinical practice for postmenopausal women with low bone density given the prevalence of both observed in otherwise healthy postmenopausal women.

Chapter 5

Associations between bone mineral density, aortic calcification and aortic stiffness in postmenopausal women

5.1 Introduction

Osteoporosis and CVD are common age-related conditions. Traditionally, the two conditions were considered unrelated and their coexistence was attributed to independent age-related processes. However, increasing biological and epidemiological evidence suggests that there are age-independent associations between BMD, VC [359], atherosclerotic burden and CV-related events, morbidity and mortality [358, 361, 366, 396-398, 402, 404, 408, 409, 420, 470, 607-613]. Tankó et al in 2003 examined low BMD at the hip as a marker of VC in elderly women [360] and reported a significant negative association between BMD at the proximal femur with the severity of AC ($r = -0.12-17$, $p < 0.001$). A further study by the same group in 2005 investigated the relationship between CVD and OP in a large cohort of over 2500 postmenopausal women and reported women with OP at the spine have a four-fold increase in the risk of a CV event compared to those with osteopenia [361]. Studies have also assessed the relationship between AAC and fracture risk and fracture status, for example, a study by Schulz et al examined the association between AAC and the risk of osteoporotic fractures in over 2000 postmenopausal women and concluded that AAC is a strong predictor of low BMD and fragility fractures and that the rate of bone loss correlates with the progression of AAC [359]. More recently a large study assessed the relationship between AAC and vertebral fracture and reported increased levels of AAC as measured by lateral lumbar radiographs and lateral VFA scans are associated with prevalent vertebral fractures, independent of BMD, age, BMI and non-vertebral fracture history [614]. Several hypotheses have been proposed to explain the age-independent association between OP and CVD. It has been suggested that CVD and bone mineralisation share common risk factors including physical activity, smoking, menopause and hypertension which may simultaneously promote or inhibit atherosclerosis and demineralisation of bone [355, 360, 402, 615], however, the association between OP and CVD remains even after correcting for these risk factors in many studies. Further suggestions for the link between the two conditions involve the presence of common pathophysiological mechanisms involving hormones and

inflammatory cytokines. Oestrogen deficiency is a major determinant of age-related bone loss and fracture risk in both men and women [84, 616] and may be involved in the pathogenesis of both atherogenesis and bone loss, either directly by the expression of oestrogen receptors on osteoblasts, osteoclasts [617] and VSMCs [618, 619], or through modulation of other factors including cytokines [620] and oxidized lipids [618]. The relationship between oestrogen deficiency and CV risk is well established [621], however observational studies have shown that postmenopausal women who receive HRT have a lower rate of CVD and cardiac death than those not receiving HRT [622, 623]. Conversely, prospective studies have shown that HRT may actually increase the risk of CV events including MI and CHD-related death in postmenopausal women [624-626]. Reasons for the contradictory results are unclear, although it has been suggested that age, pre-existing CVD and/or risk, when HRT was initiated, the type of HRT given (i.e. conjugated equine oestrogen with progestin), dosage, and the thromboembolic properties of oestrogen and progestin [620, 627-630] may influence CV outcomes. Proinflammatory cytokines including interleukin 6 (IL-6) and tumour necrosis factor (TNF) both play an important role in bone metabolism and CVD [631]. Interleukin 6 is secreted by osteoblasts in order to stimulate osteoclast differentiation and is also produced by smooth muscles cells in the tunica media of blood vessels and has been shown to stimulate the inflammatory processes involved in atherosclerosis [632]. Tumour necrosis factor has been shown to stimulate bone resorption in vitro [633], and is also a key cytokine in the recruitment and activation of inflammatory cells to the vessel wall [634]. It has also been suggested there is a causal association between OP and CVD whereby one condition may be contributing to the other [367, 399].

Arterial stiffening has long been associated with the ageing and elderly and is known to be accelerated by conventional CV risk factors including hypertension, diabetes, and hypercholesterolemia and also end stage renal failure in CKD [478, 635], and is strongly predictive of future CVD-related events [636]. Animal and human studies suggest that VC may be an important determinant of arterial stiffness [313, 595, 596, 637]. For example, animal models, have shown warfarin and vitamin K [637], and vitamin D and nicotine [595] induced medial calcification to be associated with arterial stiffness, decreased elastin and increased collagen content [313, 595, 596, 637]. Human studies have also shown that warfarin and vitamin K are associated with increased calcification [638] have demonstrated PWV is higher in renal patients with medial and intimal VC [313] and a study by Odink et al reported a positive association between PWV and AC in a cohort of over 690 elderly, healthy subjects [639]. It has not yet been confirmed whether the association between calcification and arterial stiffness simply reflects an

association between atherosclerosis and arterial stiffness. However, studies have shown increased arterial stiffness relates to calcified, echogenic plaques but not to lipid-rich non-calcified plaques [571, 640]. There is the suggestion that VC may occur simultaneously with bone demineralisation, with a shared relationship between OP and arterial stiffness explaining the epidemiological association with OP and CV-related events [361, 366, 395, 641].

A number of studies have assessed the association between arterial stiffness and BMD in both high risk populations with CKD and lower risk, healthy populations [383, 395, 480, 561, 571, 640, 641], but the findings have been inconsistent, with studies reporting a significant negative relationship between PWV and BMD [480], while others have found no relationship at all [383, 561, 571]. The inconsistent results reported may be explained by the different imaging techniques used to measure BMD. Dual-energy x-ray absorptiometry is still the most widely used method to determine BMD in clinical practice. Quantitative computed tomography is considered a superior technique for measuring BMD, assessing fracture risk and monitoring changes in BMD, but is not routinely utilised due to high radiation exposure, high cost and lack of scanner accessibility. Dual-energy x-ray absorptiometry provides 2-dimensional areal BMD measurements and the projected area analysed in DXA contains both cortical and trabecular bone however, PA DXA scans of the lumbar spine will also include considerable amounts of cortical bone from the spinal processes and articular facets and are patient size dependent [36]. Furthermore, AAC is a well-recognised artefact when performing posteroanterior DXA scans of the lumbar spine [365, 642], which can attenuate or mask the relationship between PWV and lumbar spine BMD. Quantitative computed tomography on the other hand provides 3-dimensional volumetric BMD measurements that allow the integral measurement of bone which is not size dependent. In summary, there is strong evidence of a biological link between OP and CVD, but the mechanisms involved remain poorly understood [398]. Moreover, studies assessing the association between arterial stiffness and BMD have reported conflicting results.

Table 5.1 provides a summary of previous studies evaluating relationships between BMD, AC/VC, fracture and arterial stiffness that were used to compare and correlate with the results from the present study.

Table 5.1 Overview of previous studies evaluating associations between BMD, AC/VC, fracture incidence and risk and arterial stiffness.

Author (year)	Population (n)	Mean age (yrs)	Imaging method	Outcome Measure	Study outcome
Tanko (2003) ^[360]	PM (>900)	71.3	DXA Rad	BMD VC	Sig assoc
Hak (2000) ^[371]	PM (>200)	49.0	DXA Rad	BMD VC	Sig – assoc
Kiel (2001) ^[472]	GP (>550)	>53	Rad	BMD VC	Sig – assoc
Toussaint (2008) ^[561]	CKD (48)	64.5	DXA CT PWV	BMD VC AS	Sig – assoc BMD/VC
Aoyagi (2001) ^[378]	GP (>500)	>62	DXA Rad	BMD VC	No assoc
Vogt (1997) ^[643]	PM (>2000)	>65	DXA	BMD VC	No assoc
Farhat (2006) ^[374]	GP (>400)	>49	CT	BMD VC	Sig – assoc
El Maghraoui (2013) ^[614]	PM (>900)	60.9	DXA	VC Frac	AAC assoc with Frac
Flipon (2012) ^[644]	PM (>660)	80.0	DXA Rad	VC Frac	No assoc
Schulz (2004) ^[359]	PM (>2000)	>50	DXA CT	VC Frac	Assoc AC & Frac
Frost (2008) ^[383]	PM (54)	63.4	DXA PWV	BMD AS	Sig – assoc BMD/AC
Raggi (2007) ^[480]	CKD (149)	>46	DXA QCT PWV	BMD AS	Sig – assoc BMD/PWV
Cecelja (2011) ^[571]	GP (900)	>57	DXA PWV	BMD AS	Sig assoc BMD/PWV

CKD, chronic kidney disease; GP, general population; PM, postmenopausal; AS, arterial stiffness; Frac, fracture.

5.1.1 Study aim

The rationale for this part of the study was to investigate the associations between AC, arterial stiffness and BMD in a low risk population of postmenopausal women by evaluating relationships between BMD at the lumbar spine and hip (i) AAC measured using lateral VFA scans, (ii) VC measured using CT and (iii) carotid-femoral PWV as a measure of aortic stiffness.

5.2 Study population

The study population consisted of 428 healthy, ambulatory postmenopausal women with a mean age of 62 years who had attended the Osteoporosis Unit to take part in a large cross-sectional study as described in Section 2.4, Chapter 2. Subjects that were found to have liver or renal disease defined as CKD stage 3 or greater [595], endocrine or metabolic bone disorders (other than postmenopausal OP) and hyperparathyroidism were excluded from analysis. Four subjects found to have primary hyperparathyroidism and 12 subjects that were found to have CKD-3 were excluded from the study.

5.3 Materials and methods

5.3.1 Anthropometric measurements, blood pressure and medical history

All subjects had height, weight, blood pressure and BMI measured, and a medical history was recorded for each subject, obtained using questionnaires and by personal interviews as described in Section 2.13, Chapter 2.

5.3.2 Laboratory assessments

All subjects had whole blood samples collected under fasting conditions for the measurement of lipid, bone and renal profiles (Section 2.14, Chapter 2).

5.3.3 Measurement of BMD

All subjects had bone density measured at the lumbar spine, total hip and femoral neck using DXA (Hologic, Bedford MA) as described in Sections 2.8.1 and 2.8.2, Chapter 2. Bone mineral density results were classified using the world health organisation (WHO) classification for BMD [640] as described in Section 1.3, Chapter 1. FRAX scores were also calculated using the FRAX fracture risk assessment tool (Section 1.3, Chapter 1).

5.3.4 Lateral VFA scans

Three hundred and thirty (77%) of the 428 subjects had single-energy lateral VFA (IVA-HD) scans of the lumbar spine using the Hologic Discovery QDR 4500 densitometer (Hologic, Bedford MA) (Section 2.9, Chapter 2).

5.3.5 Semi-quantitative assessment of AAC

Abdominal aortic calcification was assessed on the lateral VFA scan images by one rater (a clinical scientist [SE]), blinded to subject demographics and BMD results with only the subject's subject number included in the scan header file. Abdominal aortic calcification was evaluated using the 24-point scoring method as described in Section 2.9.1, Chapter 2 in which both the location and the severity of calcified deposits at each lumbar vertebral segment were evaluated [596].

5.3.6 CT imaging

One hundred and twelve of the 428 subjects underwent non-contrast non-diagnostic, ungated CT scans of the chest and abdomen. The scans were performed using the Philips Precedence 16-slice SPECT/CT helical scanner (Philips Healthcare, Best, Netherlands) with a field of view (FOV) of approximately 50cm to include the IA, AA, TA and CA. Scan images were reconstructed into contiguous 3mm transverse slices for viewing on the image analysis workstation, and all images were viewed and analysed using the semi-automated hybrid viewer function on Hermes Gold™ imaging software as described in Section 2.11.1, Chapter 2.

5.3.7 Quantitative assessment of calcification on CT

Scans were assessed by one rater (a clinical scientist [SE]), blinded to subject demographics, BMD results and lateral VFA AAC scores, with scans identified only by the subjects study number as described in Section 2.12, Chapter 2.

5.3.8 PWV ultrasound

Three hundred and fifty one (82%) of the 428 subjects had carotid to femoral PWV performed to measure arterial stiffness using the SphygmoCor (AtCor Medical LTD, Australia) PWV system (Section 2.10, Chapter 2). Measurements were made by one clinical scientist (SE), and any readings which did not conform to the internal quality checks provided by the SphygmoCor software were rejected and subsequently repeated. Measurements were made in triplicate, with mean PWV values used for data analysis (Chapter 2).

5.4 Statistical analysis

Study population characteristics are presented as the mean and standard deviation (SD) unless otherwise stated. To test variation between groups, analysis of variance (ANOVA) was used for continuous variables and the chi-square test used for categorical variables. In this study measures of calcification were treated as categorical rather than continuous variables due to data being highly positively skewed. VFA and CT acquired measures of AAC and VC respectively were categorised as no, moderate or high calcification with none defined as zero calcification, high as $\geq 75^{\text{th}}$ percentile and moderate as $> \text{zero}$ and $< 75^{\text{th}}$ percentile for this study population. Results for PWV were categorised into tertiles for low, moderate and high PWV scores (Table 5.2).

Table 5.2 Categories of scores for measures of VC and PWV.

	Zero * 0	Moderate >0 and < 75 th percentile	High $\geq 75^{\text{th}}$ percentile
Lateral VFA 24-point score (AU)	0	1 - 3	≥ 4
CT volume score (cm³)	0	0.001 - 0.375	≥ 0.376
PWV (m/s)	≤ 8.0	8.1 – 9.3	≥ 9.4

* defined as low for PWV scores in the lowest tertile.

Bone mineral density results were categorised into diagnostic groups as normal, osteopenic or osteoporotic using T-scores according to the WHO criteria [640]. Multinomial regression analysis was used to assess associations between BMD and AAC measured using lateral VFA, BMD and VC measured using CT and BMD and arterial stiffness measured using PWV on the total study cohort and on subjects who did not use calcium and vitamin D supplements. This regression approach was chosen instead of ordinal logistic regression because the assumption of proportional odds was not met; that is the relationships between pairs of outcome groups were statistically different. Unadjusted, age-adjusted and risk factor-adjusted models were performed to assess associations between each measure of calcification and PWV with BMD. Variables included in the risk factor-adjusted model included age, BMI, MAP, total-cholesterol, total triglycerides, smoking, physical activity and hypertension. Lumbar spine, femoral neck and total hip BMD SD scores were entered into the regressions models separately to avoid collinearity between these variables.

5.5 Results

The study population characteristics are presented in Table 5.3. Subjects had a mean age of 62 years (range 50 to 81) with no prior history of CV events. Fifty five percent (n=235) of subjects had a family history of CV disease, 13% (n=55) of subjects had confirmed hypertension with 10% (n=42) taking antihypertensive medication and 25% (n=106) of subjects reported taking prescribed calcium and vitamin D supplements for an average duration of 5 years at the time of the investigations. Subjects on prescribed calcium and vitamin D supplementation reported taking an average dose of 1200 mg of calcium carbonate and 800 IU of colecalciferol daily. Twenty percent (n=86) had normal BMD, 58% (n=247) were classified as osteopenic and 22% (n=95) as osteoporotic according to the WHO criteria as described in Section 1.3, Chapter 1. Sixty percent (n=198) of the 330 subjects that had lateral VFA scans had evidence of AAC and 78% (n=87) of the 112 subjects that had CT scans had VC. Twenty three percent (n=98) of the 428 lateral VFA scan images were excluded due to poor image quality resulting from profuse bowel gas, obesity and/or image artefacts. Statistical differences were observed between characteristics from the total study cohort and those in the CT sub-study group. CT sub-study group subjects were older than the total study population with a mean age of 63 years and had higher HDL-cholesterol levels than the total study cohort (Table 5.3).

Table 5.3 Study population characteristics.

	Total cohort (n= 428)	CT sub-study (n=112)	p-value
	Mean (SD)	Mean (SD)	
Age (years)	61.7 (6.4)	63.0 (7.1)	0.048
Time since menopause (years)	12.4 (7.4)	13.1 (7.3)	0.308
Height (m)	1.62 (0.07)	1.62 (0.07)	0.885
Weight (kg)	65.8 (12.0)	64.8 (9.6)	0.273
BMI(kg/m ²)	24.9 (4.4)	24.7 (3.7)	0.590
MAP (mmHg)	93.6 (10.0)	93.9 (9.4)	0.758
Cholesterol (total) (mmol/l)	6.0 (0.9)	6.1 (1.0)	0.171
HDL-cholesterol (mmol/l)	2.0 (0.5)	2.1 (0.5)	0.040
LDL-cholesterol (mmol/l)	3.5 (0.9)	3.6 (0.9)	0.516
Triglycerides (mmol/l)	1.0 (0.5)	1.0 (0.4)	0.338
eGFR (ml/min)	79.7 (14.6)	80.4 (15.3)	0.773
Serum calcium (mmol/l)	2.4 (0.1)	2.4 (0.1)	0.859
Lumbar spine BMD (g/cm ²)	0.893 (0.1)	0.875 (0.1)	0.121
Lumbar spine T-score	-1.4 (1.1)	-1.5 (1.0)	0.138
Femoral neck BMD (g/cm ²)	0.711 (0.1)	0.697 (0.09)	0.133
Femoral neck T-score	-1.2 (0.9)	-1.3 (0.8)	0.053
Total hip BMD (g/cm ²)	0.839 (0.1)	0.828 (0.1)	0.272
Total hip T-score	-0.8 (0.8)	-0.9 (0.8)	0.072
% Osteoporosis	22	12	0.123
FRAX Score major %	9.8 (5.7)	10.7 (6.0)	0.102
FRAX Score hip %	1.6 (2.5)	2.1 (2.9)	0.062
Dyslipidaemia %	25	5	0.061
Hypertension %	13	2.3	0.528
Family history of CVD %	55	17	0.536
Current smoker (%)	6	1	0.357
Former smoker (%)	28	8	0.245

Frequency plots for AAC, volume score and PWV are shown in Figure 5.1.

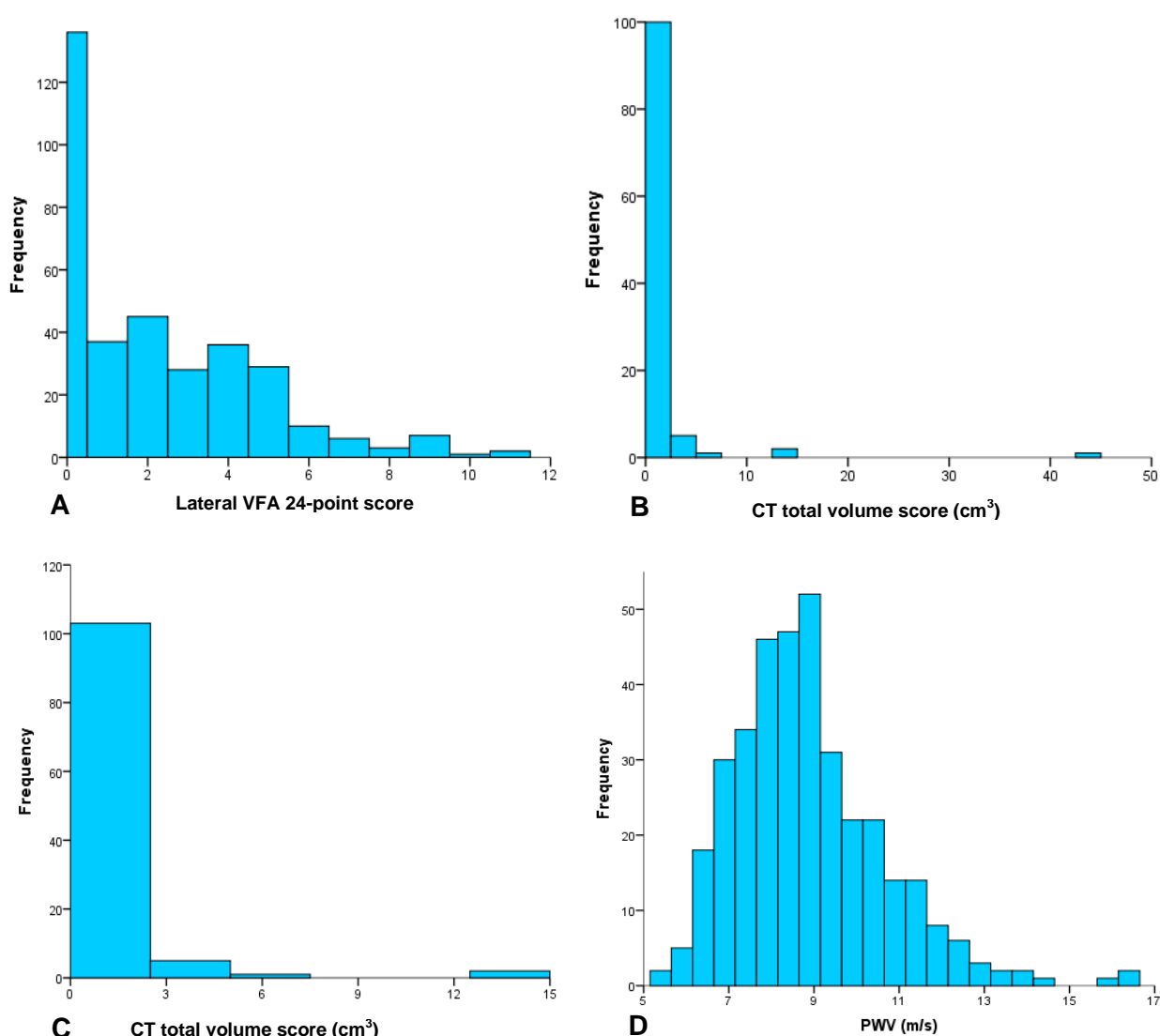


Figure 5.1 Frequency distribution histograms for AAC measured using the lateral VFA 24-point score (A), VC measured using the CT total volume score (B) and the CT total volume scores with outlier removed. Frequency histogram D shows the distribution of PWV scores. Tests for normality showed each of the scores to be positively skewed with p-values of <0.001.

The prevalence and extent of AAC as measured using the lateral VFA 24-point score and VC measured using the CT volume score according to age categories are presented in Figure 5.2A and B and the distribution of PWV scores according to age categories are presented in Figure 5.2C. Thirty three percent of subjects had moderate AAC with scores between 1 and 3 AU, and 27% of subjects had high AAC with scores ≥ 4 AU (Figure 5.2A). Thirty three percent of subjects had moderate VC with scores between

0.001-0.375cm³, and 33% had high VC with scores ≥ 0.376 cm³, with the extent of VC increasing with age (Figure 5.2B). Thirty five percent of subjects had moderate PWV between 8.1 and 9.3 m/s and 31% had high PWV with scores of ≥ 9.4 m/s which also increased with age (Figure 5.2C).

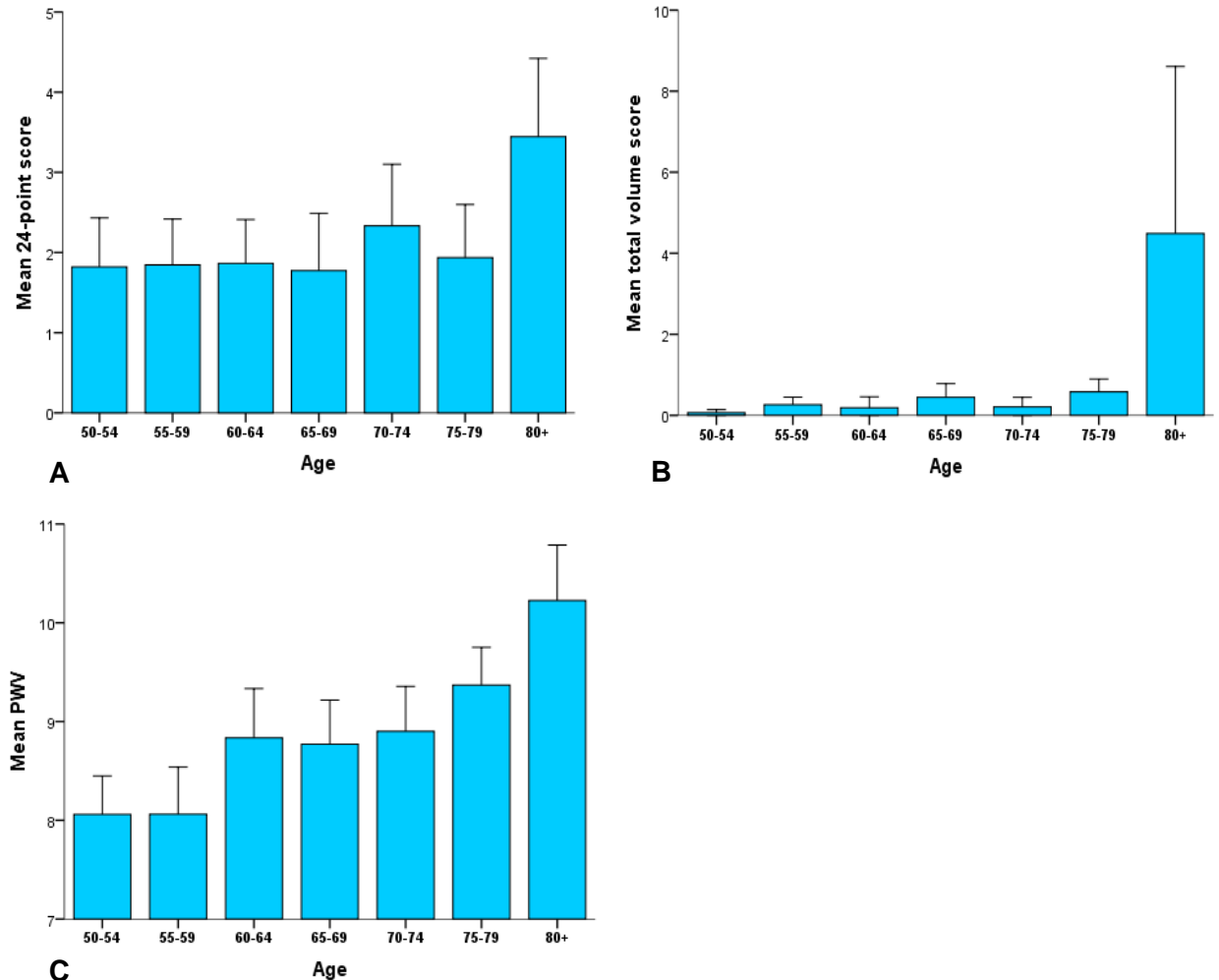


Figure 5.2 Bar graphs showing the distribution on mean (A) AAC, (B) VC and (C) PWV scores according to categories of age. The cyan box shows mean AAC, VC and PWV scores with error bars indicating the 95%CI.

5.5.1 Relationship between BMD with AAC as measured by lateral VFA

The study characteristics for each category of AAC, as measured using VFA are shown in Table 5.4. There were statistical differences in subject characteristics between the three AAC score categories. Subjects with high AAC scores were older than those with moderate and zero AAC, with a longer time since menopause. In addition, subjects with high AAC had lower eGFR results than those with zero and moderate AAC. There were

no significant differences with BMD at the lumbar spine, femoral neck or total hip between AAC score categories.

Table 5.4 Characteristics for categories of AAC as measured by the lateral VFA 24-point score.

	No AAC (Score = 0) n=132	Moderate AAC (Score = 1-3) n=109	High AAC (Score = ≥ 4) n=89	<i>p-value</i>
	Mean (SD)	Mean (SD)	Mean (SD)	
Age (years)	60.7 (5.6)	61.2 (6.4)	63.2 (7.1)	0.011
Time since menopause (yrs)	11.7 (6.8)	11.6 (7.4)	14.2 (7.4)	0.017
Height (m)	1.62 (0.07)	1.62 (0.07)	1.64 (0.06)	0.101
Weight (kg)	66.8 (9.5)	67.1 (12.2)	69.2 (13.2)	0.273
BMI(kg/m ²)	25.7 (4.0)	25.5 (4.4)	25.8 (4.7)	0.831
MAP (mmHg)	94.7 (9.4)	92.1 (10.2)	94.8 (10.9)	0.083
Cholesterol (total) (mmol/l)	6.1 (1.0)	6.0 (0.9)	6.1 (0.8)	0.818
HDL-cholesterol (mmol/l)	2.0 (0.5)	1.9 (0.5)	2.0 (0.5)	0.450
LDL-cholesterol (mmol/l)	3.6 (0.9)	3.6 (0.9)	3.6 (0.7)	0.918
Triglycerides (mmol/l)	1.0 (0.5)	1.1 (0.5)	1.1 (0.4)	0.239
eGFR (ml/min)	82.3 (15.2)	80.6 (13.3)	76.8 (13.2)	0.022
Serum calcium (mmol/l)	2.4 (0.2)	2.4 (0.09)	2.4 (0.09)	0.773
Lumbar spine BMD (g/cm ²)	0.897 (0.1)	0.891 (0.1)	0.898 (0.1)	0.906
Lumbar spine T-score	-1.4 (1.1)	-1.4 (0.9)	-1.3 (1.3)	0.840
Femoral neck BMD (g/cm ²)	0.716 (0.09)	0.723 (0.1)	0.724 (0.1)	0.802
Femoral neck T-score	-1.2 (0.8)	-1.1 (0.9)	-1.2 (0.9)	0.837
Total hip BMD (g/cm ²)	0.850 (0.1)	0.853 (0.1)	0.848 (0.1)	0.693
Total hip T-score	-0.8 (0.9)	-0.7 (0.8)	-0.8 (0.9)	0.707
FRAX Score major %	8.9 (5.2)	9.7 (6.0)	10.7 (5.7)	0.087
FRAX Score hip %	1.3 (1.8)	1.6 (3.0)	1.9 (2.9)	0.184
History of fracture (%)	30	28	35	0.076
Current smoker (%)	5	6	2	0.278

Results of the multinomial regression analysis assessing the relationship between BMD and AAC in are presented in Table 5.5. There were no significant associations between moderate or high AAC with BMD at the lumbar spine, femoral neck or total hip in the unadjusted, age-corrected or risk-factor adjusted regression models (Table 5.5). Triglycerides, age, smoking status and physical activity were independently associated with an increased risk of AAC (OR=2.06 (1.07-3.97), $p=0.029$), (OR=1.07 (1.02-1.12), $p=0.002$), (OR=4.16 (1.03-16.7), $p=0.044$), (OR=3.25 (0.91-11.6), $p=0.047$). The

relationship between BMD and AAC remained the same when analysis was limited to subjects who did not take prescribed calcium and vitamin D (data not shown). The use of calcium and vitamin D and fracture history were not found to be independent predictors of AAC when included in the regression models.

Table 5.5 Results of multinomial logistic regression models for AAC as measured using the lateral VFA 24-point score (AU). Unadjusted, age-adjusted and risk factor-adjusted ORs (95% CI) per 1 SD decrease in lumbar spine, femoral neck and total hip BMD.

	No AAC (0)	Moderate AAC (1 - 3)	High AAC (≥ 4)
<i>Lumbar Spine BMD</i>			
Unadjusted	1.00	0.94 (0.73-1.22)	1.02 (0.78-1.34)
Age-adjusted	1.00	0.96 (0.74-1.24)	1.07 (0.82-1.41)
Risk factor-adjusted*	1.00	0.96 (0.73-1.26)	1.04 (0.78-1.39)
<i>Femoral neck BMD</i>			
Unadjusted	1.00	1.05 (0.81-1.36)	1.01 (0.77-1.33)
Age-adjusted	1.00	1.07 (0.83-1.40)	1.09 (0.83-1.44)
Risk factor-adjusted*	1.00	1.17 (0.88-1.56)	1.17 (0.87-1.58)
<i>Total hip BMD</i>			
Unadjusted	1.00	1.01 (0.78-1.32)	0.87 (0.65-1.14)
Age-adjusted	1.00	1.03 (0.79-1.35)	0.94 (0.70-1.25)
Risk factor-adjusted*	1.00	1.11 (0.83-1.50)	0.94 (0.61-1.29)

*adjusted for age, BMI, MAP, total cholesterol, triglycerides, smoking status, PAS and hypertension.

5.5.2 Relationship between BMD with VC as measured by CT (sub-study)

Study characteristics for each category of VC measured using the CT total volume score are shown in Table 5.6. Subjects with high VC scores were significantly older than those with moderate or no VC with a mean age of 68 years, and had a longer time since menopause. Furthermore, subjects with high VC scores had significantly lower eGFR and significantly higher FRAX scores than those with zero calcification and moderate VC scores. Subjects with high VC scores had sustained significantly more fractures in the past than subjects with only moderate VC scores or zero calcification. There was no significant difference in lumbar spine, femoral neck or total hip BMD between VC score categories.

Table 5.6 Characteristics for categories of VC as measured by CT using the volume score (cm³).

	No VC (0) n=38	Moderate VC (0.001-0.375) n=37	High VC (≥0.376) n=37	p-value
	Mean (SD)	Mean (SD)	Mean (SD)	
Age (years)	58.6 (5.2)	62.3 (6.0)	68.3 (7.7)	<0.001
Time since menopause (yrs)	9.4 (5.8)	12.3 (6.9)	18.4 (6.7)	<0.001
Height (m)	1.64 (0.07)	1.62 (0.07)	1.60 (0.07)	0.080
Weight (kg)	62.8 (8.7)	65.6 (9.4)	65.5 (10.5)	0.460
BMI(kg/m ²)	23.3 (3.0)	24.9 (3.5)	25.7 (4.4)	0.069
MAP (mmHg)	93.3 (9.2)	94.1 (9.5)	94.1 (9.9)	0.933
Cholesterol (total) (mmol/l)	6.0 (1.0)	6.2 (1.0)	6.1 (1.0)	0.524
HDL-cholesterol (mmol/l)	2.2 (0.5)	2.1 (0.5)	2.0 (0.6)	0.616
LDL-cholesterol (mmol/l)	3.3 (0.9)	3.6 (0.9)	3.5 (0.9)	0.427
Triglycerides (mmol/l)	0.9 (0.4)	1.1 (0.5)	1.1 (0.3)	0.492
eGFR (ml/min)	81.8 (10.2)	84.3 (15.9)	73.3 (15.6)	0.022
Serum calcium (mmol/l)	2.4 (0.1)	2.4 (0.2)	2.4 (0.1)	0.089
Lumbar spine BMD (g/cm ²)	0.883 (0.1)	0.864 (0.1)	0.896 (0.1)	0.530
Lumbar spine T-score	-1.5 (1.1)	-1.7 (1.0)	-1.4 (1.2)	0.580
Femoral neck BMD (g/cm ²)	0.679 (0.07)	0.710 (0.1)	0.689 (0.1)	0.358
Femoral neck T-score	-1.5 (0.6)	-1.2 (0.9)	-1.5 (0.9)	0.277
Total hip BMD (g/cm ²)	0.821 (0.09)	0.834 (0.1)	0.824 (0.1)	0.848
Total hip T-score	-1.0 (0.7)	-0.9 (0.9)	-1.0 (0.7)	0.733
FRAX Score major %	8.4 (4.5)	10.4 (6.2)	13.3 (6.2)	0.012
FRAX Score hip %	1.2 (1.1)	1.9 (2.9)	3.3 (3.7)	0.022
History of fracture (%)	13	46	38	0.048
Current smoker (%)	8	8	3	0.641

Statistical differences in characteristics were also observed between calcium and vitamin D users compared to those that did not take supplements for score categories of VC (data not shown). Calcium and vitamin D users with high VC were significantly older ($p=0.017$), had higher systolic blood pressure ($p=0.047$), had higher lumbar spine BMD values ($p=0.024$) and lumbar spine T-scores ($p=0.038$), and had the highest PWV scores ($p=0.009$). Results of the multinomial logistical regression analysis assessing the relationship between BMD and VC are presented in Table 5.7. There were no significant associations between BMD at the lumbar spine, femoral neck or total hip with moderate or high VC in the unadjusted, age-corrected or risk-factor adjusted regression models (Table 5.7). Age was independently associated with an increased risk of moderate and

high VC in the fully adjusted models for LSBMD (OR=1.11 (1.00-1.22), $p=0.033$), (OR=1.26 (1.11-1.43), $p<0.001$), and FNBMD (OR=1.28 (1.13-1.45), $p<0.001$) respectively. There was no significant change in the relationship between BMD and VC in subjects who did not take prescribed calcium and vitamin D with logistical regression (data not shown). Calcium and vitamin D use and fracture history were not found to be independent predictors of VC.

Table 5.7 Results of multinomial logistic regression models for VC as measured using the total volume score (cm³). Unadjusted, age-adjusted and risk factor-adjusted ORs (95% CI) per 1 SD decrease in lumbar spine, femoral neck and total hip BMD.

	No VC (0)	Moderate VC (>0 - 0.375)	High VC (≥ 0.376)
<i>Lumbar Spine BMD</i>			
Unadjusted	1.00	0.84 (0.52-1.37)	1.15 (0.63-1.97)
Age-adjusted	1.00	0.91 (0.53-1.56)	1.24 (0.64-2.40)
Risk factor-adjusted*	1.00	0.89 (0.50-1.57)	1.30 (0.63-2.70)
<i>Femoral neck BMD</i>			
Unadjusted	1.00	1.40 (0.83-2.38)	1.13 (0.61-2.07)
Age-adjusted	1.00	1.55 (0.89-2.70)	1.41 (0.71-2.80)
Risk factor-adjusted*	1.00	1.34 (0.77-2.35)	1.26 (0.59-2.70)
<i>Total hip BMD</i>			
Unadjusted	1.00	1.13 (0.69-1.85)	1.03 (0.58-1.80)
Age-adjusted	1.00	1.29 (0.76-2.18)	1.33 (0.69-2.54)
Risk factor-adjusted*	1.00	1.11 (0.62-2.00)	1.23 (0.56-2.70)

*adjusted for age, BMI, MAP, total cholesterol, triglycerides, smoking status, PAS and hypertension.

5.5.3 Relationship between BMD and PWV

Study characteristics for each PWV score category are shown in Table 5.8. There were a number of significant differences between PWV score categories (Table 5.8).

Table 5.8 Characteristics for score categories of PWV

	Low PWV (≤ 8.0) n=92	Moderate PWV (8.1-9.3) n=167	High PWV (≥ 9.4) n=92	p-value
	Mean (SD)	Mean (SD)	Mean (SD)	
Age (years)	58.6 (5.1)	61.3 (5.8)	65.4 (6.5)	<0.001
Years since menopause	9.3 (5.8)	12.2 (7.2)	16.3 (7.8)	<0.001
Height (cm)	1.63 (0.06)	1.62 (0.07)	1.61 (0.06)	0.176
Weight (kg)	61.8 (9.1)	64.5 (10.4)	67.2 (8.9)	0.002
BMI(kg/m ²)	23.4 (3.1)	24.3 (3.6)	25.8 (3.3)	<0.001
MAP (mmHg)	87.9 (8.2)	92.5 (8.0)	98.9 (9.5)	<0.001
Cholesterol (total) (mmol/l)	5.9 (0.9)	6.2 (0.9)	6.2 (0.9)	0.026
HDL-cholesterol (mmol/l)	2.1 (0.5)	2.1 (0.5)	1.9 (0.5)	0.080
LDL-cholesterol (mmol/l)	3.4 (0.8)	3.6 (0.9)	3.7 (0.9)	0.022
Triglycerides (mmol/l)	0.9 (0.4)	1.1 (0.5)	1.1 (0.5)	0.164
eGFR (ml/min)	80.6 (13.1)	81.9 (15.8)	78.0 (12.7)	0.140
Serum calcium (mmol/l)	2.4 (0.06)	2.4 (0.1)	2.4 (0.09)	0.222
Lumbar spine BMD (g/cm ²)	0.899 (0.1)	0.892 (0.1)	0.881 (0.1)	0.610
Lumbar spine T-score	-1.3 (1.2)	-1.4 (1.0)	-1.5 (1.0)	0.725
Femoral neck BMD (g/cm ²)	0.718 (0.09)	0.711 (0.09)	0.702 (0.1)	0.569
Femoral neck T-score	-1.2 (0.9)	-1.2 (0.9)	-1.3 (0.9)	0.663
Total hip BMD (g/cm ²)	0.839 (0.1)	0.840 (0.1)	0.839 (0.1)	0.990
Total hip T-score	-0.8 (0.8)	-0.8 (0.8)	-0.9 (0.9)	0.996
FRAX Score major (%)	8.2 (4.6)	9.8 (5.5)	11.6 (6.5)	<0.001
FRAX Score hip (%)	1.0 (0.9)	1.6 (2.2)	2.5 (3.8)	<0.001
Fracture history (%)	26	31	34	0.519
Current smoker (%)	9	6	3	0.299

Statistical differences in characteristics were also observed between calcium and vitamin D users compared to those that did not take supplements for PWV score categories (data not shown). Calcium and vitamin D users with high PWV scores were significantly older ($p<0.001$), weighed more ($p=0.010$) had higher BMI ($p=0.048$) and had higher SBP ($p<0.0001$) and MAP ($p=0.022$). Calcium and vitamin D users with high PWV scores also had a trend for high VC scores although this failed to reach statistical significance with ANOVA. Results of the multinomial regression analysis assessing the relationship between BMD and PWV are presented in Table 5.9. There were no significant associations between BMD at the lumbar spine, femoral neck or total hip with

moderate or high PWV in the unadjusted, age-corrected or risk-factor adjusted regression models (Table 5.9). Age and MAP were independently associated with an increased risk of moderate and high PWV in the fully-adjusted models for LSBMD (OR=1.10 (1.04-1.17), $p<0.001$), (OR=1.06 (1.02-1.11), $p<0.001$), FNBMD (OR=1.09 (1.03-1.15), $p<0.001$), (OR=1.06 (1.02-1.10), $p<0.001$) and THBMD (OR=1.09 (1.03-1.16), $p<0.001$), (OR=1.07 (1.03-1.10), $p<0.001$) respectively. BMI was also found to be independently associated with an increased risk of high PWV. The relationships between BMD and PWV remained the same with subjects who did not take prescribed calcium and vitamin D (data not shown), and the use of calcium and vitamin D was not an independent predictor of increased arterial stiffness. Fracture history was not found to be an independent predictor of increased PWV.

Table 5.9 Results of multinomial logistic regression models for PWV. Unadjusted, age-adjusted and risk factor-adjusted ORs (95% CI) per 1 SD decrease in lumbar spine, femoral neck and total hip BMD.

	Low PWV (≤ 8.0)	Moderate PWV (8.1 - 9.3)	High PWV (≥ 9.4)
<i>Lumbar Spine BMD</i>			
Unadjusted	1.00	0.94 (0.74-1.23)	0.86 (0.64-1.16)
Age-adjusted	1.00	1.02 (0.78-1.34)	1.04 (0.75-1.44)
Risk factor-adjusted*	1.00	0.94 (0.70-1.25)	0.82 (0.55-1.20)
<i>Femoral neck BMD</i>			
Unadjusted	1.00	0.93 (0.72-1.20)	0.84 (0.63-1.13)
Age-adjusted	1.00	1.00 (0.76-1.30)	1.06 (0.77-1.47)
Risk factor-adjusted*	1.00	0.92 (0.68-1.24)	0.82 (0.55-1.22)
<i>Total hip BMD</i>			
Unadjusted	1.00	1.01 (0.78-1.31)	0.99 (0.74-1.33)
Age-adjusted	1.00	1.14 (0.86-1.51)	1.36 (0.97-1.90)
Risk factor-adjusted*	1.00	0.99 (0.72-1.34)	0.66 (0.63-1.44)

*adjusted for age, BMI, MAP, total cholesterol, triglycerides, smoking status, PAS and hypertension.

5.6 Discussion

The rationale for the present study was to investigate the associations between BMD with measures of VC and aortic stiffness as evidence from cross-sectional and prospective studies suggest age-independent associations between BMD and VC [356, 360, 371, 436, 472] - an established surrogate of atherosclerosis and CV risk [645, 646].

However, some studies have reported conflicting results [378, 643, 644, 647, 648]. The results of the present study showed no significant associations between BMD measured at the lumbar spine, femoral neck or total hip with AAC measured using lateral VFA scan images, and VC measured using CT.

A number of studies have shown an inverse relationship between BMD and VC in low risk populations of postmenopausal women [360, 371, 472], and in high risk populations with CKD and ESRD [561]. However, populations with renal disease are predisposed to high incidence and extent of VC and adynamic bone disease. Moreover, some studies have found no relationship between BMD and VC [378, 643, 647, 648]. A study by Farhat et al evaluated the relationship between BMD and AC both measured using CT, in a population of over 400 middle-aged women as part of the 'Study of Women's Health Across the Nation' (SWAN) and reported volumetric BMD (vBMD) to be associated with high AC in both unadjusted and adjusted regression models [374]. The present study was of a similar design to the SWAN study with a similar sample size, and due to the highly skewed data, measures of AAC and VC were treated as categorical variables and categorised as zero, moderate and high AC scores. However, unlike the study described above, the results of the present study were not significant. The different radiological techniques used to measure VC and AC may partially explain the inconsistency with results reported from studies assessing relationships between BMD and VC. Computed tomography is considered the 'gold-standard' for measuring VC whereas the identification and quantification of AAC on lateral lumbar radiographs and lateral VFA scan images is subjective and may be greatly affected by poor image quality and/or image artefacts which could result in the misinterpretation of AAC. Furthermore, vBMD measured at the lumbar spine using CT is considered to be superior compared with DXA areal BMD measurements as the method eliminates the risk of including overlying AC from the AA.

Results from the present study showed statistically significant differences in subject characteristics between AAC scores measured using lateral VFA and VC measured using CT, largely driven by the difference in mean age between groups, as confirmed by the significance of age in the age-adjusted and risk-factor adjusted models (Tables 5.4 & 5.5). Subjects with higher levels of AAC and VC were older than those within the no AAC/VC and moderate AAC/VC categories; a finding consistent with those of other studies that report a prevalence of VC within the ageing population [555, 603]. Most individuals aged 60 years and older have calcification within the major arteries [556], which is significantly associated with CVD mortality [339]. A recent study by El

Maghraoui et al of over 900 postmenopausal women demonstrated that the prevalence and severity of AAC increases with age [614].

The present study found subjects with high VC measured using CT also had higher major osteoporotic ($p=0.011$), and hip ($p=0.021$) FRAX scores compared to subjects with moderate or no VC (Table 5.6). These findings are not surprising considering one of the major determinants of FRAX is age and moreover, subjects with higher quantities of VC are older than those without. However, the present study found no independent associations between fracture history with AAC and VC unlike the study by El Maghraoui et al discussed above that reported extensive AAC to be associated with prevalent vertebral fractures independent of age, BMD, BMI and non-vertebral fracture history [614]. Another recent study also found no association between AC and incident osteoporotic fracture [644]. Conversely, a prospective study by Schulz et al in 2004, evaluated AC and the risk of osteoporotic fractures in a population of over 2000 healthy, postmenopausal women using CT to measure both AC and vBMD, with data divided into quartiles based on the percentage change in AC, and results from the study reported women with the highest increase in AC are predisposed to OP and fragility fractures [359].

A further aim of the present study was to investigate the associations between aortic stiffness and BMD but the study found no significant associations between BMD at the lumbar spine, femoral neck or total hip with aortic stiffness. Several studies have assessed the association between arterial stiffness and BMD in both high risk populations with renal disease and lower risk, healthy populations [383, 480, 561, 571] but the findings have also been inconsistent. The use of different imaging modalities for measuring BMD may, in part, explain the contradictory findings in studies evaluating relationships between BMD and aortic stiffness measured using PWV. For example, the study by Raggi et al used QCT to estimate BMD values and reported a significant inverse relationship between BMD and PWV [480], while other studies using DXA as a measure of BMD report no association between BMD and PWV [561, 571]. Although DXA is the most widely used method used for measuring BMD, QCT is considered a superior method as it provides a 3-dimensional volumetric measurement of trabecular and cortical bone density in g/cm^3 compared with 2-dimensional areal BMD expressed in g/cm^2 obtained by DXA [649, 650]. The application of QCT is limited by a higher radiation exposure as previously highlighted compared with DXA, nevertheless, BMD estimates by QCT have the advantage of not being biased by posterior elements of the vertebra including the laminae and spinous processes, severe degenerative changes, or AAC anterior to the lumbar vertebra [651-653]. Results of the present study also showed

age and MAP to be the strongest determinants of PWV, which is consistent with the findings of other studies [571, 606, 654-656].

The present study showed the use of prescribed calcium and vitamin D was not associated with AAC, VC or PWV. Calcium and vitamin D supplements are widely used for the prevention and treatment of low bone density and OP in postmenopausal women who have low levels of these nutrients in their diets [657]. The consumption of calcium and vitamin D supplements has increased in recent years with data from the 2003–2006 NHANES suggesting that predominantly middle-aged to elderly educated white women, in particular, use calcium supplements [658], which reflect the population demographics in the present study. Research has shown in addition to the benefits to bone, calcium and vitamin D may confer other health benefits such as a reduced risk of CVD [659, 660] and related CV risk factors, including hyperlipidemia [661, 662], hypertension [663, 664] and obesity [665, 666] type 2 diabetes [667, 668], certain cancers [669-671] and mortality [672, 673]. Conversely, more recently there has been much attention on the possible harmful effects of calcium intake on vascular outcomes and mortality in healthy adults [657, 674-677], and it has been suggested that calcium supplementation at doses used in clinical practice accelerates the calcification of vascular tissue [678, 679]. The mechanisms by which the use of calcium supplements increases the risk of CV events are open to speculation however, a number of studies have reported an acute increase in serum calcium following 500–1,000mg doses of supplemental calcium which may be contributing to the development of calcification within the vascular wall [678, 680-682]. Moreover, Rubin et al reported carotid plaque thickness to be greater in older subjects with higher serum calcium levels [683] and Bolland et al reported that for each 0.1 mmol/L increase in serum calcium, the likelihood of AAC increases by 23% in a population of normal older women [684].

The present study had several limitations. Only a sub-group of 112 out of the 428 subjects had CT measurements of VC. The quantification of AAC on single-energy lateral VFA scan images is highly subjective with image quality greatly affected by overlying bowel gas, patient size and/or image artefacts precluding adequate image analysis. Bone mineral density was measured using the widely used method of DXA, however the addition of BMD results measured using QCT may have strengthened the relationships between BMD, VC and arterial stiffness as observed by others. It should also be noted in the present study only 106 subjects of the total cohort were reported to be taking prescribed calcium and vitamin D supplements, with only 23 of those subjects having had CT scans to measure VC. Moreover, the use of supplementation in the present study was self-reported, and the study did not account for treatment compliance

and the additional dietary intake of calcium and vitamin D. Finally, the present study cohort consisted of healthy, postmenopausal women and therefore may not represent the male population or other populations with chronic disease.

5.7 Conclusion

The present study did not find any significant associations between BMD and measures of VC or aortic stiffness as reported in other studies. Moreover, this study did not find the use of prescribed calcium and vitamin D supplements to be independently associated with VC, AAC or aortic stiffness. Due to the highly subjective nature of quantifying AC using single-energy lateral VFA scans the method cannot be considered reliable enough to measure any associations between BMD and AAC and results may not be comparable to those obtained using dual-energy VFA scans. Furthermore the measurement of vBMD measured at the lumbar spine is considered superior compared with DXA that provides only areal BMD, and therefore further observational and prospective studies using the 'gold-standard' of CT to measure both vBMD and VC are required to fully establish the relationships between BMD, AC/VC, aortic stiffness and fracture risk and also to further explore the relationships between calcium and vitamin D supplementation with AAC, VC and aortic stiffness.

Chapter 6

Associations between regulators of bone remodelling dickkopf-1 (Dkk1) and sclerostin, and BMD, VC and arterial stiffness in postmenopausal women

6.1 Introduction

The relationship between OP, VC and subsequent CV morbidity and mortality has been demonstrated in numerous epidemiological studies [361, 369, 376, 685], and it is clear that there are causal associations between low BMD and CVD. Prospective studies have shown AC to be related to bone loss and an increase in fracture risk [359, 686], and animal studies have shown that many important biochemical factors involved in the regulation of bone and mineral metabolism may also be implicated in the pathogenesis of VC. For example, several murine knockout models of genes that regulate bone formation have led to new insights into the pathogenesis of VC [687]. However, many of these studies focus mainly on the osteoprotegerin/receptor activator of nuclear factor- κ B (RANK)/RANK ligand triad, the FGF-23/Klotho axis, and circulating osteogenic cells [688]. Mice lacking matrix Gla protein (MGP) have extensive calcification within the aorta and coronary arteries [416]. A study by Bucay et al demonstrated OPG-null mice developed medial and sub-intimal calcifications that were apparent within two weeks, and these mice also developed severe OP [410]. Fibroblast growth factor-23 (FGF-23) is a bone hormone that promotes phosphate excretion and inhibits vitamin D biosynthesis in the kidney. Klotho, which acts as both a membrane associated protein and a secreted mediator, is responsible for the kidney-specific action of FGF-23 [689]. Studies have demonstrated the deletion of Klotho in mice leads to accelerated ageing, osteopenia and extensive VC [690, 691]. An increase in the number of circulating osteoprogenitor cells was observed in OPG-null mice, and was found to correlate with the amount of calcium in the vessels [692]. Despite the significance of these findings, the clinical importance of the results remains inconclusive. There is increasing interest in signalling pathways that are involved in both bone metabolism and the development of VC and it has been suggested that the examination of these pathways may provide further insight into the association and co-existence of bone and vascular disease. The Wnt signalling pathway is an essential physiological regulator involved in the embryonic development and maintenance of various organs and tissues in the body, and the Wnt/ β -catenin signalling pathway plays an important role in bone formation by the regulation of osteoblast

proliferation and differentiation [693]. Wnt's and their receptors have also been identified as having defined roles in vascular development [442]. Although the exact role of the Wnt pathway in vascular development is relatively understudied, research is providing new and convincing data which support the role of this signalling pathway as a possible causal link between OP and VC. The Wnt signalling pathway is regulated by a number of secreted inhibitors including Dickkopf-1 (Dkk1) and Sclerostin. Dickkopf-1 has been shown to be expressed in atherosclerotic plaques [444], which is suggestive of a role of Wnt/ β -catenin signalling in the development of VC [444]. However, the precise role of Dkk1 and both its association with VC and systemic effects on bone remain to be elucidated. In-vitro studies have found sclerostin, another of the Wnt inhibitors, to be expressed in calcifying VSMCs [453]. Recent studies by Mödder et al and Mirza et al have demonstrated that circulating levels of sclerostin are higher in postmenopausal women compared to premenopausal women, and sclerostin may be a potentially important mediator of oestrogen effects on bone turnover [694, 695]. A recent study by Thambiah et al reported a positive association between circulating sclerostin, age and bone mass in pre-dialysis CKD patients [696] and a further study by Mödder et al also found circulating sclerostin to be positively associated with age and bone mass [697]. It is clear that the Wnt/ β -catenin pathway has an important role in vascular pathology as well as in bone biology and evidence confirming the presence of Wnt inhibitors Dkk1 and sclerostin within blood vessels provides the rationale for investigating whether they have a protective role or are directly involved in the pathogenesis of VC.

A summary of previous animal and human studies evaluating relationships and associations between BMD, AC/VC, arterial stiffness with Dkk1 and that have been used to compare and correlate with the results from the present study are presented in Table 6.1.

Table 6.1 Overview of previous animal and human studies evaluating associations between BMD, AC/VC and arterial stiffness with Dkk1 and sclerostin.

Author (year)	Population (n)	Mean age (yrs)	Imaging method	Outcome measure	Study outcome
Hampson (2013) ^[445]	PM (146)	61.5	DXA PWV	BMD SCLE Dkk1 AS	+ assoc SCLE & BMD/PWV
Cejka (2012) ^[698]	CKD (76)	62.0	DXA	BMD	+ assoc SCLE & BMD
	GP (45)	62.0	pQCT	SCLE	
Mödder (2011) ^[697]	GP (>600)	>20	DXA	TB-BMC	+ assoc SCLE & TB-BMC
			pQCT	SCLE	
Thambiah (2012) ^[696]	CKD (77)	57.0	DXA	BMD	- Assoc Dkk1 & BMD
				Dkk1	
Butler (2011) ^[699]	GP (36)	48.2	DXA	BMD	- assoc Dkk1 & BMD
				Dkk1	
Register (2013) ^[700]	DM-2 (450)	55.4	vBMD	BMD	No assoc
				Dkk1	
				SCLE	
Zhu (2011) ^[453]	In-vitro	-----	-----	VC	>Expres SCLE in VSMC
	Ex-vivo (An)			SCLE	>VC
Okawa (1998) ^[701]	Ex-vivo (An)	-----	-----	VC	
				SCLE	
Shao (2005) ^[443]	Ex-vivo (An)	-----	-----	VC	>Expres SCLE in VC
	Experimental			SCLE	
Claes (2013) ^[702]	CKD (154)	59.7	Rad	VC	-----
				Dkk1	
Viaene (2013) ^[703]	HD (100)	68.0	NS	VC	+ assoc Dkk1 & BMD
				Dkk1	
Brandenburg (2013) ^[704]	HD (64)	61.0	CT	VC	+ assoc SCLE & valvular VC
				Dkk1	
Ueland (2009) ^[452]	In-vitro (An)	-----	-----	VC	>Expres Dkk1 in NCAP
	Human (127)	NS		Dkk1	

HD, haemodialysis; CKD, chronic kidney disease; GP, general population; CT, computed tomography; AS, arterial stiffness; PM, postmenopausal; DM-2, type 2 diabetes mellitus; pQCT, peripheral quantitative computed tomography; vBMD, volumetric bone mineral density; Dkk1, dickkopf-1; SCLE, sclerostin; NS, not stated; An, animal; TB-BMC, total body bone mineral content; Expres, expression; NCAP, non-calcified atherosclerotic plaque; Rad, radiographs.

6.1.1 Study aim

The rationale for this part of the study was to investigate the relationships between circulating Wnt inhibitors, Dkk1 and sclerostin, with BMD, aortic stiffness and VC in healthy postmenopausal women. Relationships between inhibitors of the Wnt signalling pathway Dkk1 and sclerostin with BMD and VC may provide further evidence of their

role in the pathogenesis of OP and VC, both of which are frequently seen in the ageing female population.

6.2 Study population

The study population consisted of a sub-set of 106 healthy, ambulatory postmenopausal women with a mean age of 62 years who had attended the Osteoporosis Unit to take part in a large cross-sectional study as previously described in Section 2.4, Chapter 2. Only women who had serum sclerostin and Dkk1 levels measured were included in this part of the study. Subjects had BMD measurements of the lumbar spine and hip, PWV ultrasound measurements to assess aortic stiffness, CT scans of the chest and abdomen and lateral VFA scans to quantify AC. Subjects also had routine laboratory assessments measurements of sclerostin and Dkk1.

6.3 Materials and methods

6.3.1 Anthropometric measurements, blood pressure and medical history

All subjects had height, weight, BMI and BP measured as described in Chapter 2. A detailed medical history was recorded for each subject, obtained using questionnaires and by personal interviews as described in Chapter 2.

6.3.2 Laboratory assessments

All subjects had whole blood samples collected under fasting conditions for the measurement of lipid, bone and renal profiles (Chapter 2). Serum samples were stored frozen at -70° and measurements of serum sclerostin and serum Dkk1 were performed as one batch at the end of the study. The sclerostin and Dkk1 samples were analysed at the Bone Biochemistry Laboratory (Sheffield Teaching Hospitals NHS Foundation Trust). Serum sclerostin was measured using a commercially available enzyme linked immunosorbent assay (ELISA) (Biomedica Gruppe, Wien, Austria). The intra-assay CV was 5% at a mean sclerostin concentration of 54.0 pmol/l and the minimum detection limit of each assay was 2.6 pmol/l. Serum Dkk1 was measured using ELISA (Biomedica Gruppe, Wien, Austria) according to the manufacturer's instructions. The minimum detection limit was 0.38 pmol/l and the intra-assay CV was 7% and 8% at Dkk1 concentrations of 19.1 pmol/l and 10.1pmol/l respectively. One subject with primary hyperparathyroidism was excluded from the study (Chapter 2).

6.3.3 Measurement of BMD

All subjects had bone density measured at the lumbar spine, total hip and femoral neck in addition to whole body composition scans to measure total body-bone mineral content (TB-BMC) using DXA (Hologic, Bedford MA) as described in Sections 2.8.1 and 2.8.2, Chapter 2. Bone mineral density results were classified using the world health organisation (WHO) classification for BMD [53] as described in Section 1.3, Chapter 1. FRAX scores were also calculated using the FRAX fracture risk assessment tool (Section 1.3, Chapter 1).

6.3.4 Lateral VFA scans

All subjects had single-energy lateral VFA (IVA-HD) scans of the lumbar spine using the Hologic Discovery QDR 4500 densitometer (Hologic, Bedford MA) (Section 2.9, Chapter 2). Scans were obtained using the established local protocol for the evaluation of vertebral deformity which was modified to include the lumbar region only as described in Section 2.9, Chapter 2. Eighty four (80%) of the 106 lateral VFA scans were successfully evaluated. Twenty two (20%) of the 106 lateral VFA scans were unevaluable due to overlying bowel gas and abdominal obesity affecting image quality and therefore precluding adequate image analysis.

6.3.5 Semi-quantitative assessment of AAC

Abdominal aortic calcification was assessed on lateral VFA scan images by one clinical scientist (SE), blinded to subject demographics and BMD results with scans identified only by the subjects study number. Scans were analysed using the semi-quantitative 24-point scoring method [564] as described in Section 2.9.1, Chapter 2.

6.3.6 CT imaging

One hundred and six subjects underwent non-contrast non-diagnostic, un-gated CT scans of the chest and abdomen as described in Section 2.11.1, Chapter 2.

6.3.7 Quantitative assessment of VC

Scans were assessed by one clinical scientist (SE), identified only by the subjects study number. Each 3mm scan slice was analysed separately and calcified deposits were

defined by the rater as any area greater than 1mm^2 with a HU of ≥ 130 , and calcified deposits were recorded according to anatomical location and deposit number as described in Section 3.3.2 in Chapter 3. Calcification was quantified using three established VC scoring methods as described in Section 2.12, Chapter 2.

6.3.8 Measurement of arterial stiffness

All 106 subjects had carotid-femoral PWV performed to measure aortic stiffness using the SphygmoCor (AtCor Medical LTD, Australia) PWV system (Section 2.10, Chapter 2). Ninety four (89%) of the 106 subjects had successful PWV measurements. Pulse wave velocity measurements were not possible on 12 subjects due to large amounts of adipose tissue precluding an adequate reading from the femoral artery. Measurements were made by one clinical scientist (SE), and any readings which did not conform to the internal quality checks provided by the SphygmoCor software were rejected and subsequently repeated. Measurements were made in triplicate, with mean PWV values used for data analysis. The reproducibility of measurements of PWV has been previously described in Chapter 4.

6.4 Statistical analysis

Study population characteristics are presented as the mean and standard deviation (SD) unless otherwise stated. Analysis of variance was used to test for differences between categories of BMD, VC, AAC and PWV. To allow for multiple comparisons between categorical groups, ANOVA was used to calculate statistical significance between groups with a Bonferroni correction to control for Type-1 errors. The Kolmogorov-Smirnov test was used to assess normality of distribution for measures of VC including lateral VFA 24-point scores, CT volume scores and PWV. Variables that were found to be positively skewed were log transformed to normalise the data distribution. Results for AAC, VC and PWV were divided into 3 tertiles as described in Chapter 5. Abdominal aortic calcification measured using the lateral VFA 24-point score was categorised as; no calcification (zero score), moderate calcification (scores 1 to 2) and high calcification (scores ≥ 3). Similarly, calcification measured using the CT volume score (cm^3) was categorised as no calcification (zero), moderate calcification (scores >0 to 0.384) and high calcification (≥ 0.385). Pulse wave velocity scores were also categorised as low (≤ 8.0), moderate (8.1 to 9.4) and high (≥ 9.5). Univariate analysis using the Pearson's or Spearman's correlation coefficient was used to explore the relationships between parametric and nonparametric variables respectively. Multivariate regression analysis

was used to assess associations between Dkk1 and sclerostin and both BMD and measures of VC correcting for confounders. Further multivariate regression analysis was performed to assess associations between sclerostin and measures of VC correcting for total body-bone mineral content (TB-BMC). Sclerostin is primarily produced by osteocytes and increased levels of circulating sclerostin may reflect increased osteocyte numbers in individuals with a higher skeletal mass, providing the rationale for including TB-BMC in the analysis. Confounding variables included age, menopause age, BMI, MAP, lipid levels, fracture history, smoking status and physical activity. P values <0.05 were considered statistically significant.

6.5 Results

The characteristics of subjects included in this study are presented in Table 6.2. Subjects had a mean age of 63 years (range 50 to 81) with no prior history of CV events. Seventy two per cent (n=76) of subjects had a family history of CV disease. Nine (9%) of subjects had confirmed hypertension, of which 7 were currently taking antihypertensive medication. Eighteen per cent (n=19) of subjects reported taking prescribed calcium and vitamin D supplements at doses used to treat OP at the time of the investigations. Of the 84 lateral VFA scans that were successfully evaluated 61% of subjects had AAC using lateral VFA. Seventy eight percent of subjects had evidence of VC confirmed using CT.

Table 6.2 Population characteristics for the total study cohort.

(n= 106)	Mean (SD)
Age (years)	62.9 (6.7)
Age at menopause (years)	49.6 (4.2)
Height (m)	1.62 (0.07)
Weight (kg)	65.5 (10.0)
BMI(kg/m ²)	24.8 (3.8)
MAP (mmHg)	93.6 (8.9)
Cholesterol (total) (mmol/l)	6.7 (1.0)
HDL-cholesterol (mmol/l)	2.1 (0.5)
LDL-cholesterol (mmol/l)	3.5 (0.9)
Triglycerides (mmol/l)	1.0 (0.4)
Sclerostin (pmol/l)	35.0 (12.2)
Dkk1 (pmol/l)	25.1 (9.2)
Lumbar spine BMD (g/cm ²)	0.884 (0.1)
Lumbar spine T-score	-1.4 (1.1)
Femoral neck BMD (g/cm ²)	0.704 (0.09)
Femoral neck T-score	-1.2 (0.8)
Total hip BMD (g/cm ²)	0.834 (0.1)
Total hip T-score	-0.8 (0.8)
Hypertension (%)	8
Family history of CVD (%)	72
History of fracture (%)	33
Current smoker (%)	1
Former smoker (%)	29

6.5.1 Associations between Sclerostin and Dkk1 with BMD

Both Dkk1 and sclerostin data were normally distributed ($p=0.16$, $p=0.118$). Linear regression showed that sclerostin and Dkk1 were not correlated ($r=0.04$, $p=0.664$). Univariate analysis revealed significant positive correlations between sclerostin and lumbar spine ($r=0.22$, $p=0.030$), femoral neck ($r=0.31$, $p=0.002$), and total hip ($r=0.27$, $p=0.006$) BMD as shown in Figure 6.1 and this association remained significant in the adjusted models (Table 6.3). Dkk1 was not associated with BMD at any site after correcting for confounders.

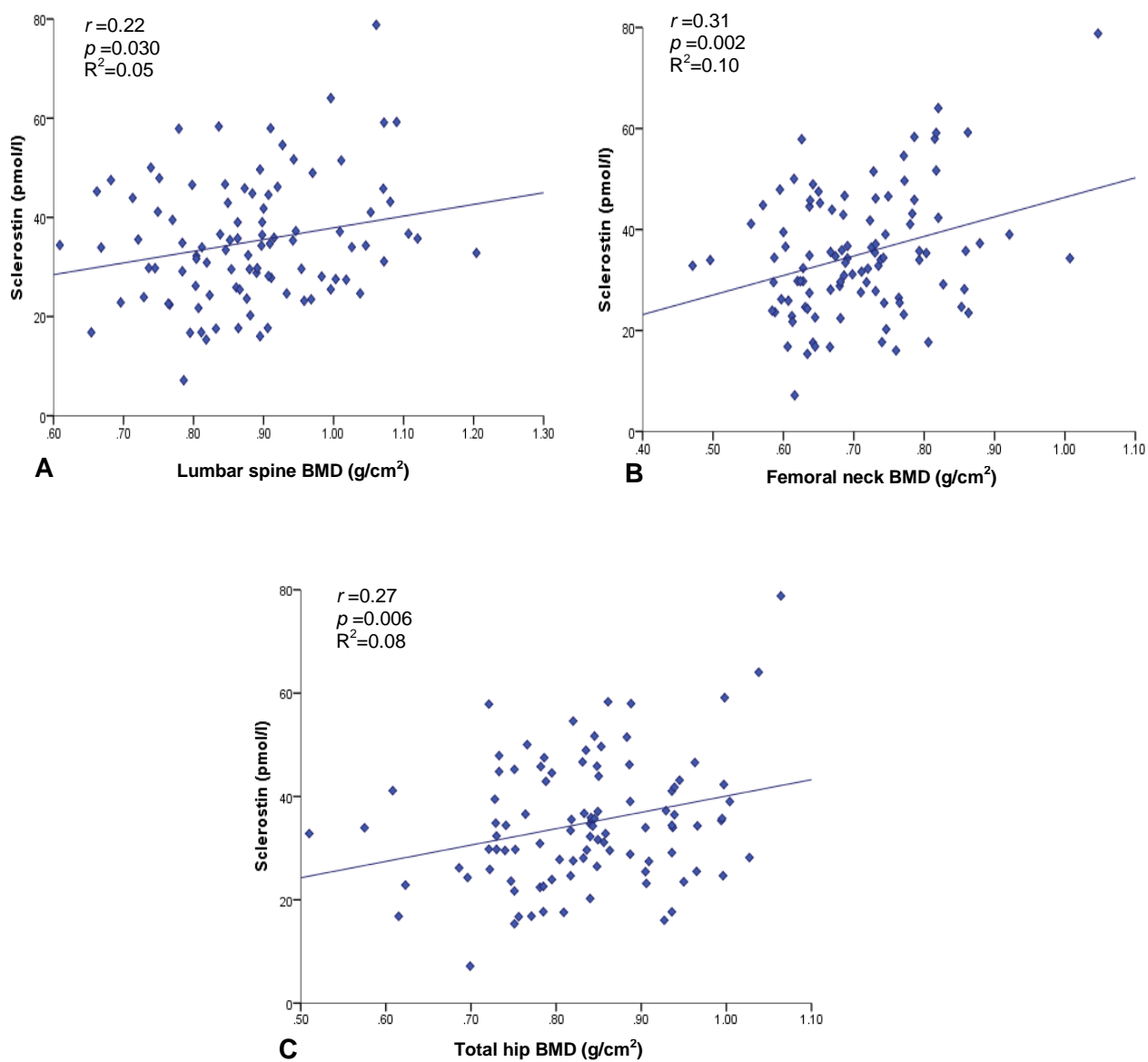


Figure 6.1 Scatterplots showing the associations between sclerostin with BMD at the lumbar spine (A), femoral neck (B), and total hip (C).

Table 6.3 Multi-linear regression analysis of lumbar spine, femoral neck and total hip BMD as the dependent variables and Dkk1 and sclerostin as independent variables.

Variables	LSBMD β -coefficient	<i>p</i> -value	FNBMD β -coefficient	<i>p</i> -value	THBMD β -coefficient	<i>p</i> -value
Age (years)	-0.228	0.032	-0.188	0.068	-0.215	0.034
BMI (kg/m ²)	0.309	0.011	0.204	0.077	0.271	0.015
Menopause age	0.021	0.827	0.099	0.297	0.157	0.092
Physical activity	0.402	0.001	0.054	0.640	0.005	0.965
Smoking status	0.124	0.228	0.169	0.085	0.167	0.083
Fracture history	0.061	0.559	-0.154	0.128	-0.206	0.037
Sclerostin (pmol/l)	0.296	0.006	0.328	0.001	0.281	0.004
Dkk1 (pmol/l)	0.006	0.951	-0.126	0.185	-0.115	0.216

6.5.2 Associations between vascular calcification, Sclerostin and Dkk1

There were no significant associations between either sclerostin or Dkk1 with AAC or VC following univariate analysis. Following adjustment for confounders multi-linear regression did not reveal any significant associations between sclerostin and Dkk1 with AAC, with age being the only variable positively associated with AAC (Table 6.4).

Table 6.4 Multi-linear regression analysis of log AAC measured using lateral VFA and log VC measured using the CT volume score as the dependent variables and Dkk1 and sclerostin as independent variables.

Variables	AAC β -coefficient	<i>p</i> -value	VC β -coefficient	<i>p</i> -value
Age (years)	0.387	0.035	0.640	<0.001
BMI (kg/m ²)	0.103	0.579	-0.035	0.766
Menopause age	0.164	0.292	-0.044	0.677
Physical activity	0.191	0.245	-0.052	0.649
Smoking status	0.105	0.521	0.093	0.364
Fracture history	-0.025	0.882	-0.075	0.497
Total cholesterol (mmol/l)	0.267	0.104	-0.180	0.110
Total triglycerides (mmol/l)	0.063	0.725	0.039	0.734
Sclerostin (pmol/l)	0.132	0.392	-0.006	0.957
Dkk1 (pmol/l)	0.142	0.410	-0.153	0.156

There was no significant association between sclerostin and TB-BMC following univariate analysis ($p=0.090$). The multi-linear regression model was repeated with the addition of total body BMC. The associations between sclerostin and Dkk1 with AAC and

VC remained non-significant following adjustment for TB-BMC (Table 6.5). Analysis of variance revealed there were no significant variations in median sclerostin and Dkk1 values across the three AAC/VC score categories.

Table 6.5 Multi-linear regression analysis of log AAC measured using lateral VFA and log VC measured using the CT volume score as the dependent variables and Dkk1, sclerostin and TB-BMC measured using DXA as independent variables.

Variables	AAC β -coefficient	<i>p</i> -value	VC β -coefficient	<i>p</i> -value
Age (years)	0.433	0.022	0.639	<0.001
BMI (kg/m ²)	0.033	0.853	-0.035	0.768
Menopause age	0.175	0.238	-0.044	0.681
Physical activity	0.181	0.248	-0.052	0.651
Smoking status	0.070	0.651	0.092	0.384
Fracture history	-0.018	0.915	-0.075	0.507
Total cholesterol (mmol/l)	0.285	0.071	-0.180	0.113
Total triglycerides (mmol/l)	0.142	0.424	0.039	0.736
Sclerostin (pmol/l)	0.084	0.571	-0.006	0.954
Dkk1 (pmol/l)	0.056	0.736	-0.153	0.159
TB-BMC (g)	0.321	0.036	0.003	0.982

6.5.3 Associations between arterial stiffness, Sclerostin and Dkk1

There were no significant associations between Dkk1, sclerostin and PWV in univariate or multi-linear regression analysis after correcting for confounders. Age, BMI and MAP were the only variables significantly positively associated with PWV (Table 6.6).

Table 6.6 Multi-linear regression analysis of arterial stiffness measured by PWV as the dependent variable and Dkk1 and sclerostin as independent variables.

Variables	β -coefficients	p-value
Age (years)	0.406	<0.001
BMI (kg/m²)	0.310	0.009
Menopause age	-0.140	0.159
Physical activity	0.012	0.915
Smoking status	-0.130	0.201
Fracture history	-0.025	0.808
Alcohol intake	-0.044	0.654
MAP (mmHg)	0.238	0.025
Total cholesterol (mmol/l)	-0.029	0.786
Total triglycerides (mmol/l)	-0.085	0.439
Sclerostin (pmol/l)	-0.080	0.439
Dkk-1 (pmol/l)	0.000	0.999
$R^2 = 0.35$		

Analysis of variance revealed there was a statistically significant difference in sclerostin values across the three PWV score categories ($p=0.019$). Post-hoc tests were conducted to evaluate pairwise differences among the three PWV score categories, correcting for multiple comparisons using a Bonferroni correction. Further analysis using ANCOVA to correct for variables significantly associated with PWV as determined in the regression model (i.e. age, BMI and MAP) showed no statistical difference in sclerostin values across the three PWV score categories ($p=0.395$). Analysis of variance revealed there was no significant difference in median Dkk1 values across the three PWV categories with or without inclusion of confounders.

6.6 Discussion

The rationale for the present study was to investigate the associations between circulating concentrations of the Wnt inhibitors - sclerostin and Dkk1 - with BMD, AAC, VC and arterial stiffness as the Wnt signalling pathway has been implicated in the pathogenesis of OP and may also be involved in the pathogenesis of VC. The presence of Wnt inhibitors, Dkk1 and sclerostin in and around the site of VC in blood vessels demonstrates that further research is needed to investigate their precise role and function in the development of both OP and VC. The results from the study presented here showed sclerostin to be positively associated with BMD at the lumbar spine, femoral neck and total hip as confirmed in previous studies [445, 698, 705, 706]. In

contrast, Dkk1 was not associated with BMD at any site. This study did not find any association between either sclerostin or Dkk1 with AAC, VC or with PWV.

A recent study completed by our research group using samples from a different sub-set of 140 postmenopausal women also showed that circulating sclerostin levels were positively associated with femoral neck and total hip BMD [445]. In addition, the positive relationship between sclerostin and BMD has also been reported in high risk populations with CKD [698]. The Wnt signalling pathway and its antagonists play an important role in the regulation of bone mass and osteoblastogenesis. When Wnt is absent in this pathway, β -catenin is phosphorylated and degraded, and when Wnt is present it binds to specific receptors (frizzled) and co receptors (LRP-5 and -6), leading to the inhibition of β -catenin phosphorylation and degradation. Wnt/ β -catenin signalling is not only critical for osteoblastogenesis but also for normal osteocyte function [707]. Sclerostin, a major Wnt antagonist is secreted almost exclusively by osteocytes [693, 708-710] and the binding of sclerostin to the canonical Wnt- β -catenin receptors inhibits the Wnt signalling and consequently bone formation by inhibiting the proliferation and differentiation of osteoblasts [711], and it has been suggested that this process may occur to prevent overfilling of the BMUs during the bone remodelling process, maintaining the balance between bone formation and resorption [710]. Sclerostin gene (SOST) knockout mice have increased bone formation rates and increased bone mass [712], and over expression of the SOST gene has been found to result in osteopenia [713]. A SOST gene mutation in humans, where an enhancer element (52kb) that is normally downstream of the SOST gene is deleted, leads to sclerosteosis [714] and van Buchem's disease [715] which are associated with markedly increased bone mass [716]. Furthermore, treatment of postmenopausal women with a sclerostin monoclonal antibody increases bone formation markers and therefore BMD at the lumbar spine and hip [717].

Given that sclerostin is an inhibitor of osteoblastic activity, negative associations between sclerostin and BMD would be expected. However, in the present study this was not found to be the case in agreement with other studies described above. There are currently no data in the literature on the metabolism of sclerostin, and it is unclear whether the positive relationship between sclerostin and BMD reflects an increased sclerostin production due to high osteocyte numbers in subjects with a high bone mass or due to a reduced renal degradation and clearance. Recent research has demonstrated that bone marrow and circulating blood sclerostin concentrations are highly correlated, which suggest that circulating concentrations of sclerostin may reflect

the bone marrow environment and therefore increased production is unlikely due to decreased clearance or breakdown [445, 718].

A large study by Mödder et al in 2011, found sclerostin levels to be positively associated with total body bone mineral content in addition to lumbar spine and femoral neck BMD in a large study of over 650 subjects suggesting an increased sclerostin production by osteocytes in subjects with a higher bone mass [697]. However, this was not found to be the case in the present study as there was no significant association between sclerostin levels and TB-BMC.

In contrast to the positive association observed between sclerostin and BMD, results of the present study showed no association between Dkk1 and BMD (Table 6.3). Results from studies evaluating the relationship between Dkk1 and BMD have been inconsistent, with some reporting significant negative associations between Dkk1 and BMD in renal patients [696], and in patients with OP [699], while others have reported no relationship [700] consistent with the results of the present study. Unlike sclerostin that is almost exclusively expressed by osteocytes, Dkk1 has a wider tissue distribution and is expressed by tissues including endothelial cells, neural cells and platelets among others, with the latter being considered a major contributor to circulating Dkk1 levels in recent studies [719, 720]. This will mask or attenuate any significant relationship particularly as the skeletal expression of Dkk1 is not the only contributor to circulating Dkk1 levels.

The present study found no significant association between sclerostin and AAC measured using lateral VFA or with VC measured at multiple vascular sites using CT. Furthermore, there were no significant differences in sclerostin levels with increasing levels of calcification (Table 6.4). Sclerostin is expressed in high levels by mature osteocytes and previous experimental studies have demonstrated an increased expression of sclerostin in areas of VC and in calcifying VSMC [453, 721]. For example, Zhu et al reported sclerostin to be up-regulated during VSMC calcification in vitro and also reported an increased expression of sclerostin in the calcified media of aortic tissue in NPP1-null mice [453] - ecto-nucleotide pyrophosphatase/phosphodiesterases-1 (NPP1) is a major generator of extracellular pyrophosphate which is a known VC inhibitor [722]. Further research on NPP1-null murine models showed that these mice spontaneously developed calcified articular cartilage, peri-spinal calcification, and medial AC at a young age [701]. Interestingly, these NPP1-null mice share phenotypic features with a human disease, idiopathic infantile arterial calcification [723, 724]. Furthermore, experimental studies of CMV-Msx2 transgenic mice develop extensive VC and have also shown an increased expression of sclerostin at the site of VC [443]. The expression of

sclerostin has also been found within the sub endothelial layer of the intima in pathological specimens of human aorta [725].

There are limited data regarding the association between circulating concentrations of sclerostin and VC in humans and although these studies have demonstrated both a positive and inverse relationship between sclerostin and VC, the majority have been limited to higher risk populations with renal disease and DM that have a greater incidence and severity of VC [700, 702, 703]. For example, a recent cross-sectional analysis evaluating the association between sclerostin and AC in patients with CKD showed a positive association between AC and higher sclerostin levels, although this association became inverse following multivariate analysis [702]. Another similar cross-sectional study on chronic haemodialysis patients showed a strong positive association between sclerostin and calcifying aortic heart valve disease and further demonstrated sclerostin is locally expressed in aortic valve tissue adjacent to areas of calcification [704]. Results from a pilot study evaluating associations between aortic valve calcification (AVC) and sclerostin showed increased circulating sclerostin levels to be associated with increased severity of AVC, compared with healthy controls that had lower sclerostin levels [726]. In contrast to these findings, a post-hoc analysis of data from 100 haemodialysis patients revealed that increased circulating levels of sclerostin were associated with a decrease in all-cause mortality, and authors go as far to suggest that high circulating sclerostin levels protect patients on dialysis from CV calcification [703]. It has been suggested that associations between sclerostin and VC may imply an increase in the phenotypic transition of VSMCs to osteocytes and an up-regulation of sclerostin by these transformed vascular cells during the VC process [445, 453]. Therefore circulating sclerostin concentrations may partly reflect the production and secretion by cells within the arterial wall. Thus, it can be postulated that increased production of sclerostin may simply be a protective mechanism preventing further progression of VC [445]. However, further clinical and experimental studies are required to clarify whether or not sclerostin protects against the progression of VC.

The present study did not observe any associations between Dkk1 with AAC or VC at multiple vascular sites. The recent study by our group on a different sub-set of postmenopausal women than those included in the present study showed that Dkk1 concentrations were significantly lower in those with high AAC as measured by lateral DXA [445]. The analysis of the Dkk1 samples was carried using two different laboratory locations with the present study data reported by the Bone Biochemistry Laboratory, Sheffield, whereas the Dkk1 data from the different subset of women was reported by the Department of Clinical Chemistry, St Thomas Hospital [441]. Therefore the variation

between assays may account for the different results reported here. An inverse relationship between Dkk1 and calcified plaques in higher risk patients with type-2 DM has also been reported [700]. Studies have also reported an increased expression of Dkk1 in atherosclerotic plaques and in the circulation of patients with unstable angina [452] which may reflect an increased inflammatory response, or may potentially be a protective response against the development and progression of further calcification [445]. The varying effects and interactions between Dkk1, VSMCs, endothelial cells or vascular adventitia observed in different clinical settings may also reflect differences between the study populations.

The present study found age to be positively associated with VC, which is a common occurrence within the ageing population [429]. Medial calcification is recognised as a significant contributing factor to arterial stiffness and studies have shown sclerostin to be associated with medial calcification [452, 453, 727], whereas Dkk1 appears to be closely related to intimal calcification which represents the advanced stage of atherosclerosis [728]. This suggests that a high expression of sclerostin in the presence of large quantities of medial calcification would result in increased serum sclerostin levels in subjects with a high PWV. However, the present study found no association between sclerostin and PWV even after correcting for confounders associated with arterial stiffness. Moreover, there was also no association between Dkk1 and PWV (Table 6.6). Data from the present study did show a trend for higher sclerostin levels in women with high PWV although this was not significant. Similarly, the study on a different sub-set of postmenopausal women by our group reported no significant association between sclerostin and PWV, but did observe a significant difference in sclerostin levels between high and low PWV score groups [445]. Conversely, a recent study on pre-dialysis CKD patients reported a negative association between sclerostin and arterial stiffness [695]. There are limited data in the literature regarding the associations between circulating sclerostin and Dkk1 levels with arterial stiffness in healthy, ambulatory populations, and whether arterial stiffness and its contributing factors lead to an increase in circulating sclerostin, or increased circulating sclerostin levels result in a reduction of arterial compliance and a subsequent increase in PWV remains to be determined. The present study found age to be positively correlated with PWV which was not an unexpected finding as age is considered to be a significant contributory factor responsible for the reduction in arterial compliance leading to increased PWV. Results also showed MAP and BMI to be associated with PWV which is consistent with previous studies [729, 730].

This study had several limitations. The cross-sectional design did not facilitate the assessment of causal associations between sclerostin, BMD, arterial stiffness and VC.

Furthermore, the present study did not include measurements of inflammatory markers, although it is thought inflammation may enhance the extra-skeletal expression of circulating Dkk1 [696]. The study consisted of only a small sub-set of women from the total study cohort, and women who were known to be taking lipid lowering drugs were excluded. Limitations with the imaging techniques used meant it was not possible to distinguish between intimal and medial calcification which may have allowed the assessment of both, particularly as both medial and intimal calcification can be present in areas of VC.

6.7 Conclusion

In conclusion, the present study found Wnt inhibitor sclerostin to be positively associated with BMD measured at multiple sites but no association was found between BMD and Dkk1. Furthermore this research did not find any association between VC or arterial stiffness with either sclerostin or Dkk1. This is the first study to clinically evaluate not only associations between Dkk1 and SCLE with BMD, but also AC measured using VFA scans, VC at multiple vascular sites using CT and arterial stiffness using PWV. However, further clinical studies are required to clarify the precise role of sclerostin in the pathogenesis of VC to establish whether or not sclerostin promotes or indeed protects against the progression of VC.

Chapter 7

Associations between bisphosphonates (BP), vascular calcification (VC) and aortic stiffness

7.1 Introduction

Bisphosphonates are chemically stable analogues of inorganic pyrophosphate and are potent inhibitors of osteoclastic activity, primarily used in the management of OP and also to treat metastatic bone disease to prevent osteoclast-mediated bone resorption by binding to hydroxyapatite [731]. The most widely used BPs in current clinical practice are nitrogen-containing BPs (NCBPs) including alendronate, risedronate, ibandronate and zoledronate. They inhibit the activity of farnesyl diphosphate synthase, a key regulatory enzyme in the mevalonate pathway which is the biosynthetic pathway for the production of cholesterol, other sterols and isoprenoid lipids, including farnesyl diphosphate [191, 732, 733]. Consequently BPs have similar pharmacological effects as statin therapy [734] in terms of decreasing LDL-cholesterol and increasing HDL-cholesterol levels [735, 736].

Vascular calcification exists in two distinct, but not mutually exclusive entities; intimal and medial calcification. Intimal or atherosclerotic calcifications are predominantly located in the coronary arteries and aorta and are the clinical manifestation of advanced atherosclerosis, obstructing the artery lumen increasing the risk of stroke, myocardial infarction and angina [645]. Medial or Mönckeberg's calcification on the other hand develops in the medial layer of the artery wall, and unlike intimal calcification medial calcification does not cause obstruction within the arterial lumen. It does, however, lead to increased stiffness of the elastic layer of the arterial wall, resulting in increased pulse pressure which subsequently leads to left ventricular hypertrophy, vascular dysfunction and failure [737, 738]. Although the precise mechanisms of VC remain undefined it has been suggested that VC is a consequence of a process which is similar to osteogenesis [407, 641, 739-741]. Ordinarily, mesenchymal stem cells, found in bone marrow, differentiate into adipocytes, chondrocytes, osteoblasts and VSMCs. However, oxidative stress, inflammatory cytokines or BMPs can stimulate VSMC to undergo differentiation into osteoblast-like cells, and it has been suggested this process may be critical in the development of VC [740, 741]. Studies have shown several regulatory factors, including

MGP, OPN and OPG inhibit VC and the inadequate function or loss of these inhibitors is associated with VC [416, 435, 741].

In view of the common underlying pathological mechanisms between VC and bone formation, agents that inhibit bone loss may also inhibit VC or slow the rate of progression of VC and consequently lower CV-related morbidity and mortality. Bisphosphonates have shown a consistent effect on inhibiting VC in both experimental and animal studies and research has shown that BPs inhibit experimentally induced VC in rodents through the inhibition of osteoclast-mediated bone resorption [742-744]. For example, in an experimental study by Saito et al in 2007, pamidronate was reported to inhibit VC induced by inorganic phosphate in bovine aortic smooth muscle cells, with the results showing a reduction in AC by 88%. Moreover, the study found that the inhibitory effect of pamidronate increased with higher doses of BP [744]. Price et al in 2001 conducted a series of animal studies to examine the effects of BPs on induced VC in rats and concluded that alendronate and ibandronate inhibited VC, at doses that inhibit bone resorption without affecting normal bone mineralisation [742]. Tamura et al in 2007 evaluated the effect of etidronate in rats with induced AC and reported etidronate also significantly inhibited AC at doses that did not affect bone metabolism [743]. Although the precise mechanism by which BPs inhibit VC still remains to be established, these studies provide evidence that BPs can inhibit VC and at doses equivalent to those used in clinical practice.

The effect of BPs on VC in humans is less clear, with clinical studies reporting conflicting results [509-515, 745]. To date there have been no large-scale observational studies evaluating the effects of BPs on VC and there is a lack of unequivocal evidence supporting the inhibitory effect of BPs on VC in humans. Previous studies have reported the beneficial effects of BP on VC/AC [510-513, 745], while others have shown clinical doses of BP did not decrease or slow the progression of AC [514, 515]. The conflicting findings from these studies suggest that the effects of BP on VC in humans are still inconclusive and further research is required to confirm the effects of BPs on VC and arterial stiffness.

A summary of previous clinical studies evaluating the use of bisphosphonate therapy and the effects on BMD, AC/VC, arterial stiffness and serum lipids used to compare and correlate with results from the present study are presented in Table 7.1.

Table 7.1 Overview of previous studies evaluating the effects of bisphosphonate therapy on BMD, AC/VC and arterial stiffness and serum lipids.

Author (year)	Population (n)	Mean age (yrs)	Imaging method	Outcome measure	Study outcome
Brown (2009) ^[209]	PM (1189)	64.6	DXA	P1NP CTX BMD	Sig <P1NP & CTX with BP
Adami (2000) ^[735]	PM (87)	>53	N/A	HDL LDL	IV-BP = <LDL & >HDL
Montagnani (2003) ^[736]	PD (20)	67.6	N/A	HDL LDL	IV-BP = <LDL & >HDL
Elmariah (2010) ^[734]	GP (>3600)	63.0	CT	CVC	BP assoc < prev VC in >65yrs
Tanko (2005) ^[514]	PM (>400)	>55	Rad	AC	No change AC over 3yrs
Toussaint (2010) ^[515]	CKD (51)	63.1	CT PWV	VC AS	No change in VC over 18-months
Celiloglu (2009) ^[519]	PM (72)	52.0	US	IMT	Sig <CIMT over 1yr
Igase (2014) ^[746]	PM (38)	NS	PWV	AS	Min change in PWV over 1yr

CKD, chronic kidney disease; GP, general population; AS, arterial stiffness; PM, postmenopausal; PD, Paget's disease; NS, not stated; N/A, not applicable; CVC, cardiovascular calcification; IMT, intima-media thickness; US, ultrasound; P1NP, procollagen type 1 N-terminal propeptide; CTX, C-terminal telopeptide; Rad, radiographs; BP, bisphosphonates; prev, prevalence.

7.1.1 Study aim

The aim of this part of the study was to: (1) compare VC and aortic stiffness in postmenopausal women who have been prescribed BP therapy for the treatment of OP and in women who have never used BPs using cross-sectional data; (2) evaluate the effects of alendronate treatment on AC in postmenopausal women with low BMD over 12-months using data acquired in a prospective, randomised, controlled trial (RCT).

7.2 Study population

7.2.1 Cross-sectional study

This study consisted of a population of 418 healthy, ambulatory postmenopausal women with a mean age of 62 years who had attended the Osteoporosis Unit as previously described in Section 2.4, Chapter 2. All subjects had BMD measurements of the lumbar spine, total hip and femoral neck using DXA, carotid-femoral PWV ultrasound

measurements to assess aortic stiffness, CT scans of the chest and abdomen to quantify VC, and single-energy lateral VFA scans to quantify AAC. Subjects also had blood samples collected for the measurement of bone, lipid and renal profiles and markers of bone formation and resorption. Subjects that were found to have liver or renal disease defined as CKD-3 or greater, endocrine or metabolic bone disorders and hyperparathyroidism were excluded from analysis, as described in Chapter 2. Four subjects that were found to have primary hyperparathyroidism and 12 subjects that were found to have CKD-3 were excluded from the study. Eleven subjects that had previously used but were not currently taking BPs were also excluded from this study.

7.2.2 Prospective

This study consisted of data for the first 14 patients recruited on an ongoing single-centre, 2-year, randomised, controlled, open-label trial to determine the effects of alendronate on AC and aortic stiffness. The principle inclusion criteria for enrolment onto this trial was a T-score of -2.0 or less at the lumbar spine or hip and further details regarding the inclusion and exclusion criteria can be found in Section 2.4, Chapter 2. Subjects were required to attend for a baseline and 3-month visit, followed by 5 further visits at 6-monthly intervals up to 24-months. Only 12-month data for the first 14 patients enrolled are reported here as the study is ongoing. Subjects had BMD measurements of the lumbar spine, total hip and femoral neck using DXA, carotid-femoral PWV ultrasound measurements to assess aortic stiffness, CT scans of the chest and abdomen to quantify VC, and lateral VFA scans to quantify AAC. Each subject had blood samples collected at each study visit for the measurement of bone, lipid and renal profiles and also for the measurement of markers of bone formation and resorption.

7.3 Materials and methods

7.3.1 Anthropometric measurements, blood pressure and medical history

7.3.2 Cross-sectional

All subjects had height, weight, BMI and blood pressure measured as described in Chapter 2. A detailed medical history was recorded for each subject, obtained using questionnaires and by personal interviews. Information collected included family history of CVD, family history of low trauma fractures, history of CV events, the presence of diabetes mellitus, hypertension, and hypercholesterolemia, fracture history, smoking habits, alcohol intake and physical activity levels as previously described (Chapter 2).

7.3.3 Prospective

A detailed medical history was recorded at the screening visit for each subject by the study physician, followed by a physical examination. All subjects had anthropometric and blood pressure measurements at subsequent study visits as described in Chapter 2.

7.3.4 Laboratory assessments

7.3.5 Cross-sectional

All subjects had whole blood samples collected following an overnight fast for the measurement of total cholesterol, LDL- and HDL-cholesterol and triglycerides. Subjects also had serum calcium, albumin corrected calcium, albumin, phosphate, alanine transaminase (ALT), alkaline phosphatase (ALP), bilirubin, parathyroid hormone (PTH) and estimated glomerular filtration rate (eGFR) measured to determine renal function status and exclude any subjects with liver or renal disease, endocrine or metabolic bone disorders and hyperparathyroidism from the analysis as described in Chapter 2 .

7.3.6 Prospective

Subjects had lipid and bone profiles performed at each of the study visits as described in Section 2.5.3, Chapter 2. Serum samples that were frozen and stored at -70°C were used to measure markers of bone formation and resorption at baseline, 3, 6 & 12-months including serum type 1 procollagen N-terminal (P1NP) and C-terminal telopeptide (CTX). The P1NP and CTX samples were analysed at the Bone Biochemistry Laboratory, Sheffield Teaching Hospital NHS Trust. Serum P1NP was measured using a commercially available enzyme linked immunosorbent assay (ELISA) (Roche Diagnostics, Mannheim, USA). The intra-assay CV was 2.9% at a mean P1NP concentration of 29.1ng/mL and the minimum detection limit of each assay was <5ng/mL. Serum CTX was measured using ELISA (Roche Diagnostics, Mannheim, USA) according to the manufacturer's instructions. The minimum detection limit was 0.010ng/mL and the intra-assay CV was 4.6% at CTX concentrations of 0.08 ng/mL.

7.3.7 Measurement of BMD

7.3.8 Cross-sectional

All subjects had BMD measured at the lumbar spine, total hip and femoral neck using DXA (Hologic, Inc. USA) as described in Sections 2.8.1 and 2.8.2, Chapter 2.

7.3.9 Prospective

All subjects had BMD measured at the lumbar spine, total hip and femoral neck (Sections 2.8.1 and 2.8.2, Chapter 2), at baseline, 6 and 12-months as described in Section 2.5.3, Chapter 2. Bone mineral density results were classified using the WHO classification for BMD as described in Section 1.3, Chapter 1, and fracture risk was assessed using the FRAX fracture risk assessment tool (Section 1.3, Chapter 1) which calculates a 10-year probability of a hip and major osteoporotic fracture.

7.3.10 Lateral VFA scans

7.3.11 Cross-sectional

All subjects had single-energy lateral VFA (IVA-HD) scans of the lumbar spine using the Hologic Discovery QDR 4500 densitometer (Hologic, Inc. USA) (Section 2.9, Chapter 2). Scans were obtained using the established local protocol which was modified to include the lumbar region only as described in Section 2.9, Chapter 2. Three hundred and twenty of the 418 lateral VFA scans were successfully evaluated. Of the remaining 98 (23%) lateral VFA scans 7 were unable to be evaluated due to abdominal obesity and 91 scans due to overlying bowel gas affecting image quality.

7.3.12 Prospective

Subjects taking part in the RCT had lateral VFA (IVA-HD) scans performed at baseline, 6 and 12-months as described in Section 2.5.3, Chapter 2. At baseline 13 of the 14 scans were successfully evaluated with one scan un-evaluatable due to abdominal obesity affecting image quality. At 6 and 12-months 9 and 8 scans respectively were adequately evaluated, with 5 and 6 scans respectively rejected due to overlying bowel gas precluding adequate image analysis.

7.3.13 Semi-quantitative assessment of AAC

Abdominal aortic calcification was assessed on lateral VFA scan images by one clinical scientist (SE), blinded to subject demographics and BMD results with scans identified only by the subjects' study number. Scans were analysed using the semi-quantitative 24-point scoring method as described in Section 2.9.1, Chapter 2.

7.3.14 Measurement of aortic stiffness

7.3.15 Cross-sectional

Three hundred and forty four of the 418 study subjects had successful carotid-femoral PWV performed to measure aortic stiffness using the SphygmoCor (AtCor Medical LTD, Australia) PWV system (Section 2.10, Chapter 2). Seventy four (18%) of the 418 PWV measurements were unsuccessful, with large amounts of adipose tissue precluding adequate readings from the femoral artery. Measurements were made by one clinical scientist (SE), and any readings which did not conform to the internal quality checks provided by the SphygmoCor software were rejected and subsequently repeated by the operator. Measurements were made in triplicate, with mean PWV values used for data analysis. The reproducibility of this method has been previously described (Chapter 2).

7.3.16 Prospective

Pulse wave velocity measurements were not successful for 1, 3, 3 and 5 patients at baseline, 3, 6 and 12-months respectively due to adipose tissue precluding adequate readings from the femoral artery.

7.4 Statistical analysis

7.4.1 Cross-sectional

Study population characteristics are presented as the mean and standard deviation (SD) and median and interquartile range (IQR) were stated. Differences in characteristics between the BP and control groups were determined using the student t-test for normally distributed continuous variables, the Mann-Witney test for continuous variables not normally distributed and the chi-squared test for categorical variables. P values <0.05 were considered statistically significant. The Kolmogorov-Smirnov test was used to assess normality of distribution for measures of VC including lateral VFA AAC scores and PWV. Positively skewed data including the lateral VFA 24-point AAC score and PWV score was log transformed to normalise the distribution for multivariate regression analysis. Multivariate regression analysis was used to evaluate the relationships between the severity of VC and BP use and also the duration of BP use and the severity of VC, correcting for confounders associated with VC and/or aortic stiffness including age, BMI, MAP, physical activity, smoking status, and lipid levels. Duration of BP use was divided into three categories and defined as 1-3, 4-7 and 8+ years. Prevalence ratios were assessed using logistic regression with a modified Poisson regression approach, correcting for confounders associated with VC and/or aortic stiffness, using

binary data scores of 0 for zero calcium and 1 for calcium >0, for the lateral VFA 24-point score. The threshold for PWV was defined as the median score of 8.7 m/s, with scores <8.7 assigned a binary value of 0 and scores ≥ 8.7 assigned a binary value of 1. Prevalence ratios were also calculated for AAC and PWV with thresholds above the 75th percentile of the variable.

7.4.2 Prospective

Study population characteristics are presented as the mean and standard deviation (SD) and median and interquartile range (IQR) were stated. Differences in characteristics between the BP and control group were determined using the student t-test for normally distributed continuous variables, the Mann-Witney test for continuous variables not normally distributed and the chi-squared test for categorical variables. P values <0.05 were considered statistically significant. The percentage change for outcome variables including lateral VFA 24-point score, PWV, BMD, bone turnover markers and lipid levels were calculated. The paired t-test and Wilcoxon signed rank tests were used to test for significant change in variables from baseline for each of the prospective study treatment arms. The un-paired t-test and Mann Whitney tests were used to explore the percentage change in outcome variables at each time-point between the treatment and control arms.

7.5 Results

7.5.1 Association between bisphosphonate use, VC and aortic stiffness in postmenopausal women: a cross-sectional study.

Subjects had a mean age of 62 years (range 50 to 81) with no prior history of CV events. Fifty five percent (n=230) of subjects had a known family history of CV disease. Thirteen percent (n=54) of subjects had confirmed hypertension, 42 (10%) of which were taking antihypertensive medication at the time of investigations. Seventy five percent (n=60) of subjects in the BP group reported the use of concomitant prescribed calcium and vitamin D supplements and 8% (n=27) in the control group reported taking prescribed calcium and vitamin D supplementation at doses used to treat osteopenia and OP. Seventy nine (99%) of the BP users took nitrogenous BPs (predominantly alendronate and residronate) with only one subject reportedly taking a non-nitrogenous BP (etidronate). Subjects in the BP group were significantly older with a longer time since menopause, weighed less and had a lower BMI. Furthermore, the BP group had higher total cholesterol and lower LDL-cholesterol levels compared with controls. The BP group also had lower LS, FN & TH T-scores and higher FRAX and PWV scores compared with the control group (Table 7.2).

Table 7.2 Study population characteristics for the BP groups and subjects who have never used BPs.

	Bisphosphonate Group (n=80)	Controls (n=341)	<i>p</i> -value
	Mean (SD)	Mean (SD)	
Age (years)	64.3 (7.0)	60.8 (6.0)	<0.001
Time since menopause (years)	15.4 (8.4)	11.6 (6.9)	<0.001
Height (m)	1.63 (0.07)	1.62 (0.07)	0.438
Weight (kg)	60.4 (9.1)	67.0 (12.3)	<0.001
BMI(kg/m ²)	23.0 (3.4)	25.4 (4.5)	<0.001
MAP (mmHg)	92.7 (8.7)	93.7 (10.4)	0.483
Cholesterol (total) (mmol/l)	5.8 (0.8)	6.1 (1.0)	0.017
HDL-cholesterol (mmol/l)	2.1 (0.5)	2.0 (0.5)	0.202
LDL-cholesterol (mmol/l)	3.3 (0.8)	3.6 (0.9)	0.002
Triglycerides (mmol/l)	1.0 (0.5)	1.1 (0.5)	0.357
eGFR (ml/min)	81.0 (16.8)	80.5 (14.0)	0.986
Serum calcium (mmol/l)	2.4 (0.1)	2.4 (0.1)	0.713
Lumbar spine T-score	-2.1 (1.1)	-1.2 (1.1)	<0.001
Femoral neck T-score	-1.8 (0.6)	-1.1 (0.9)	<0.001
Total hip T-score	-1.5 (0.7)	-0.7 (0.9)	<0.001
FRAX Score major (%)	13.5 (6.6)	8.8 (5.1)	<0.001
FRAX Score hip (%)	3.1 (3.1)	1.3 (2.2)	<0.001
Current smoker (%)	2	6	0.733
Hypertension (%)	19	12	0.075
Hypertension medication (%)	16	6	0.030
Family history of CVD (%)	56	55	0.837
24-point AAC score (AU)	2.6 (2.7)	2.1 (2.4)	0.105
PWV (m/s)	9.3 (2.1)	8.7 (1.6)	0.006

Figure 7.1 shows the mean lateral VFA 24-point and PWV scores for the control versus the BP group. Mean AAC and PWV scores were higher in the BP group compared to subjects who had never taken BPs (Figure 7.1). Further analysis with ANCOVA using age as a covariate showed no significant difference in PWV between the BP group and control group ($p=0.269$).

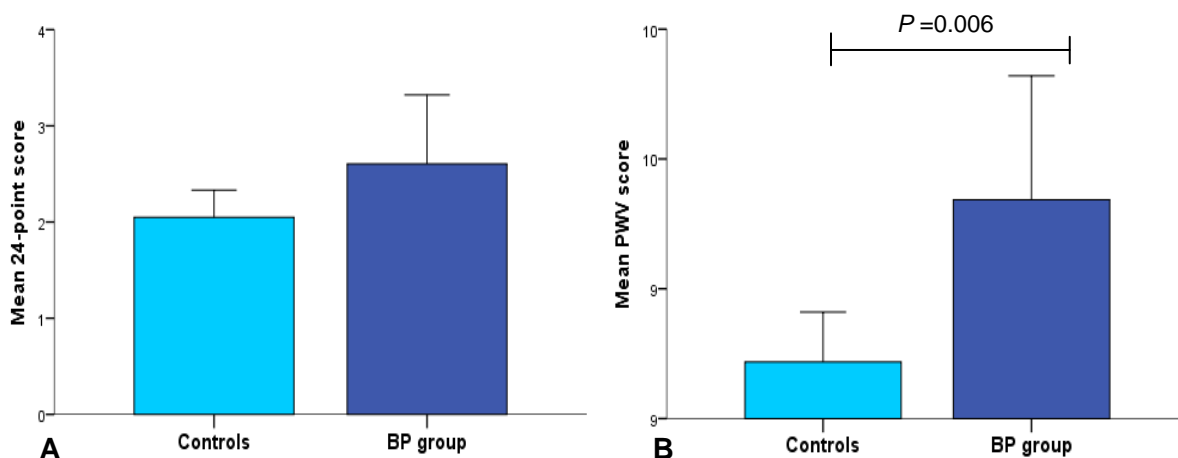


Figure 7.1 Mean 24-point AAC score (A) and mean PWV score (B) for the BP group and subjects who did not take bisphosphonates. The error bar represents the 95% CI.

AAC was present in 48% ($n=154$) of subjects in the control group and 46% ($n=37$) of subjects in the BP group. There was no statistical significance in the prevalence of AAC > 0 and PWV scores \geq the median score of 8.7 or with AAC and PWV scores above the 75th percentile between the BP group and those that had never taken BPs.

Of the 80 subjects in the BP group 39% ($n=31$) had taken BPs between 1-3 years, 28% ($n=22$) between 4-7 years and 34% ($n=27$) for 8+ years. When the subjects were split into groups according to duration of BP use no significant differences were observed in demographics, lipid levels, BMD, AAC or PWV (Table 7.3).

Table 7.3 Subject characteristics according to duration of BP treatment In years

	Category 1 (1-3yrs) n=31	Category 2 (4-7yrs) n=22	Category 3 (8+yrs) n=27	p-value
	Mean (SD)	Mean (SD)	Mean (SD)	
Age (years)	64.2 (7.0)	64.0 (7.0)	65.7 (7.2)	0.611
Time since menopause (yrs)	14.7 (9.0)	14.9 (7.6)	16.7 (8.6)	0.627
Height (m)	1.63 (0.07)	1.63 (0.07)	1.62 (0.07)	0.895
Weight (kg)	60.9 (8.9)	61.9 (8.1)	58.8 (10.3)	0.485
BMI(kg/m ²)	23.0 (3.4)	23.4 (3.4)	22.3 (3.2)	0.517
MAP (mmHg)	93.3 (8.6)	93.9 (8.1)	90.9 (8.2)	0.400
Cholesterol (total) (mmol/l)	5.8 (0.8)	5.7 (0.8)	5.9 (0.9)	0.683
HDL-cholesterol (mmol/l)	2.2 (0.5)	2.1 (0.5)	2.1 (0.5)	0.681
LDL-cholesterol (mmol/l)	3.2 (0.8)	3.2 (0.7)	3.5 (0.7)	0.266
Triglycerides (mmol/l)	1.0 (0.4)	1.1 (0.8)	0.9 (0.3)	0.636
eGFR (ml/min)	81.2 (18.6)	72.0 (12.2)	82.5 (19.0)	0.097
Serum calcium (mmol/l)	2.4 (0.2)	2.4 (0.06)	2.4 (0.08)	0.147
Lumbar spine T-score	-1.6 (1.2)	-1.1 (1.0)	-1.5 (1.0)	0.313
Femoral neck T-score	-1.3 (0.8)	-1.4 (0.8)	-1.3 (0.9)	0.827
Total hip T-score	--0.8 (0.8)	-1.1 (0.7)	-0.8 (0.8)	0.512
FRAX Score major (%)	9.1 (4.9)	10.4 (5.1)	10.1 (7.6)	0.698
FRAX Score hip (%)	1.2 (1.4)	2.0 (2.9)	1.3 (1.1)	0.301
AAC 24-point score (AU)	3.2 (3.0)	2.1 (2.7)	2.5 (2.5)	0.509
PWV (m/s)	9.1 (2.2)	9.5 (2.0)	9.5 (2.0)	0.642

Table 7.4 shows the prevalence ratio (PR) and 95% CI for the total AAC and PWV scores. Multi-logistic regression models adjusting for age, BMI, physical activity status (PAS), smoking status, total cholesterol and triglycerides demonstrated that variables associated with BP use were also significantly associated with age (Table 7.5). Two models were used as described in Section 7.4.1 with prevalence ratios calculated for AAC scores >0 and $\geq 75^{\text{th}}$ percentile of the variable. High and low PWV scores were split at the median and also at the 75^{th} percentile of the variable (Table 7.4). The results showed the use of BP treatment was not associated with an increased risk of AAC or arterial stiffness in either of the models described above. Body mass index and total cholesterol were also significantly associated with BP use in both the 24-point score and PWV models ($p=0.002$, $p=0.001$).

Table 7.4 Prevalence ratio (95%CI) for binary lateral VFA AAC and PWV scores. Results are presented as AAC >0 and median PWV. High AAC and PWV values are set at the 75th percentile of the variable.

Outcome measure	Prevalence Ratio (PR)	95% CI	p-value	Interaction with age p-value
Total 24-point AAC score				<0.001
> 0	1.00	0.60-1.66	0.988	
≥ 5	0.97	0.54-1.74	0.922	
PWV (m/s)				<0.001
≥ 8.7	1.44	0.83-2.52	0.195	
≥ 10.0	1.09	0.64-1.86	0.742	

Multivariate regression analysis showed no association between the use of BPs with AAC in the unadjusted, age-adjusted or fully-adjusted models after correcting for confounders associated with VC and arterial stiffness. Age and PAS were both significantly associated with AAC (Table 7.5). In contrast, there was a significant positive association between BP use and PWV in the unadjusted regression model ($p=0.009$) but this association did not remain in the age-adjusted model. After correcting for confounders in the fully-adjusted model, BP use was found to be significantly associated with PWV (Table 7.5).

Table 7.5 Multivariate regression analysis of log AAC measured using lateral VFA and log PWV as the dependent variable and BP use (binary) as the independent variable.

Variables	AAC	p-value	PWV	p-value
	β-coefficient		β-coefficient	
Age (years)	0.198	0.009	0.341	<0.001
BMI (kg/m ²)	0.040	0.625	0.130	0.012
Physical activity	0.161	0.031	-0.073	0.113
Smoking status	0.032	0.656	-0.029	0.521
MAP (mmHg)	0.112	0.126	0.353	<0.001
Total cholesterol (mmol/l)	0.129	0.102	0.034	0.495
Triglycerides (mmol/l)	-0.035	0.667	-0.017	0.735
Bisphosphonate use	0.029	0.708	0.105	0.031

Further multivariate analysis showed no association between the duration of BP use with either AAC or PWV (data not shown).

7.5.2 Effects of alendronate on aortic calcification and aortic stiffness in postmenopausal women: A pilot RCT

At the time of reporting only 14 subjects had completed the 12-month follow-up visit, with 10 subjects randomised to the treatment arm and only 4 subjects randomised to the control arm.

The results of the RCT are not presented here. The pilot randomised controlled study started only after the PhD project had commenced and was therefore limited by a small sample size that was not sufficiently powered to detect statistically significant change. Although the preliminary results of the RCT were not included they were considered a useful adjunct to the results of the cross-sectional study and will be published at the end of the 24-month study period.

7.6 Discussion

The rationale for the present study was to evaluate the effects of BP treatment on VC and aortic stiffness in postmenopausal women as BPs are the first line treatment for the treatment of OP and prevention of OP-related fractures [747], and accumulating evidence from experimental, animal and clinical studies suggest that BPs also have the potential to slow the progression or even induce the regression of VC [510, 537-540]. There are currently no licensed therapies which have been shown to reduce VC – an independent predictor of CVD risk – therefore a concurrent therapy that can not only treat OP but also prevent or reduce the progression of AC may be beneficial in reducing mortality resulting from CVD. Results from the cross-sectional study showed the use of BPs and the duration of BP use to be positively associated with PWV but not AAC.

Vascular calcifications can occur at two distinct sites within the vessel wall: the intima and the media. Intimal calcification occurs in the context of atherosclerosis and characterised by lipid accumulation, inflammation, fibrosis and the development of focal plaques. It is these atherosclerotic plaques that are predominantly found within the aorta [748-750]. In contrast, medial calcification, which usually occurs along the elastic lamina, is a common occurrence in patients with CKD whereby the dysregulation of calcium and phosphate metabolism is the main contribution to VC. Elevated calcium and phosphate levels have direct effects VSMCs and sequentially the VSMCs stimulate osteogenic/chondrogenic differentiation, vesicle release, apoptosis, loss of inhibitors, and extracellular matrix degradation to drive the development of VC. Research has shown BP to be a promising agent to treat both types of VC [504], although the manner

in which BPs influence its development remains to be fully established. It is known, however, that BPs have a direct effect on the arterial wall where there is a possible interaction with sub-endothelial lipid-phagocytic cells [504]. Bisphosphonates are internalised into the arterial wall by macrophage phagocytosis; a process in which macrophages ingest BPs and once phagocytosed, BPs have been reported to affect the ability of macrophages to internalise atherogenic LDL-cholesterol and the subsequent transformation into foam cells [498]. It has been suggested that BPs influence the development of VC by the reduction of serum calcium and phosphate limiting their deposition within the vascular wall. Furthermore, it has been suggested that BPs have a direct effect on calcium crystal formation by blocking hydroxyapatite nucleation and growth [751]. Results of the cross-sectional study found no association between the use of BPs and the duration of BP use and AAC measured using lateral VFA scans [Table 7.5]. Contrary to these results a recent study evaluating NCBP use and the prevalence of VC in over 3700 women showed that NCBPs were associated with a decreased prevalence of VC in subjects aged 65 years and over [734]. Possible explanations for why the present study failed to find an association between the use of BPs and AAC may be due to the small sample size compared with the significantly larger cohort in the study by Elmariah et al described above. Moreover, in the present study VC was only measured at the AA using 2-D lateral VFA imaging in only 54 subjects whereas the study by Elmariah et al evaluated coronary calcification using CT in over 3700 subjects. Both lateral VFA and CT imaging are methods which are limited by their inability to distinguish between intimal and medial calcifications and image quality can be greatly affected by patient size, which is particularly true with lateral VFA scan images where poor image quality results in a large percentage of scan images that cannot be evaluated. Moreover lateral VFA scan image analysis has a lower inter-rater agreement compared with that of CT as highlighted in preceding chapters.

Cross-sectional data from this study showed that BP users had significantly higher total-cholesterol and lower LDL- cholesterol results compared with those who had not used BPs. Nitrogen-containing BPs, including alendronate, inhibit farnesyl-pyrophosphate synthase, an enzyme in the mevalonate pathway, the site of statin action [752] and intravenous administered BP has been shown to decrease serum LDL-cholesterol levels by approximately 5% and raise HDL-cholesterol levels by 10% to 18% [735, 736]. Conversely, research evaluating the effects of alendronate on lipid metabolism and BMD in over 120 osteoporotic postmenopausal women reported no significant change in any components of the serum lipid profile following 12-months of treatment [753]. Although other studies report significant changes in serum lipids with IV- administered BP [735,

736] the data from the present study represent orally administered BPs. In addition to the effects of BP in the mevalonate pathway, IV- administered BP has also been shown to reduce inflammation by inhibiting the secretion of several inflammatory cytokines, including IL-6 and TNF [754, 755]. Vascular inflammation to some degree is a frequent concomitant of most forms of VC, therefore a reduction in inflammatory markers by BPs may confer beneficial effects in the context of inhibiting the development and progression of inflammatory diseases such as atherosclerosis and subsequently the development of VC.

Results of the cross-sectional study found a significant difference in PWV scores between BP users and those who had never used BPs [Figure 7.1], however this significant association was found to be largely driven by age. Cross-sectional data also showed a significant positive association between BP use and the duration of BP use with PWV, although this association was also confounded by age and MAP [Table 7.5] which are both significantly associated with arterial stiffness [605, 606]. Moreover, subjects taking BPs were significantly older than subjects that had never taken BPs and therefore would most likely have increased arterial stiffness. Toussaint et al evaluated changes in PWV as an outcome measure in a randomised controlled trial evaluating the effects of alendronate on VC, and the mean PWV was reported to have increased significantly throughout the 18-month study in both the alendronate and control arm [515]. Conversely, a recent study reported minimal change in arterial stiffness in osteoporotic postmenopausal women following 12-months treatment with oral alendronate [746]. Arterial stiffness is in part dependent on atherosclerotic burden and/or medial calcification, and a reduction in PWV would be expected in subjects taking alendronate due to the inhibitory effects of BP on the development of atherosclerosis and VC [321, 605].

The present study did have several limitations. Subjects who were known to be taking lipid lowering therapy were excluded, likely lowering both the incidence and extent of VC within the study population. Only a limited number of BP users were recruited to the cross-sectional study and therefore results may not represent the true effects of BP use on VC in the wider population. Bisphosphonate use and the duration of BP use was self-reported and precluded information on treatment compliance, both of which create significant limitations, and therefore there may have been over or underestimation of duration of use. The use of lateral VFA imaging to quantify AC was limited by the inability of the technique to distinguish between intimal and medial AC, and it is possible that intimal and/or medial calcification was being measured. Most of the subjects that were taking BPs were also taking adjunct calcium and vitamin D supplements, and therefore it

was not possible to separate the effects of BPs on VC from the effects of calcium and vitamin D on VC. The pilot randomised controlled study started only after the PhD project had commenced and was therefore limited by a small sample size that was not sufficiently powered to detect statistically significant change. Although the preliminary results of the RCT were not included they were considered a useful adjunct to the results of the cross-sectional study and will be published at the end of the 24-month study period.

7.7 Conclusion

Results of the cross-sectional study presented here did not show any significant association between BP use with AAC or arterial stiffness. Large-scale RCTs are required to evaluate the vascular effect of BPs to establish whether they can provide a concurrent therapy that not only treats OP and reduces fracture risk, but may also prevent the progression or decrease VC in subjects that have an increased risk of CV morbidity and mortality.

Chapter 8

Conclusion and further work

8.1 Summary

The principle objective of this research project was to investigate the relationship between osteoporosis and aortic calcification in postmenopausal women, during which the study has provided an extensive evaluation of not only the associations between VC within the aorta and at other vascular sites with aortic stiffness, BMD and regulators of bone remodelling, but also the imaging techniques that can be used to quantify vascular and aortic calcification not only in clinical practice but also for research purposes.

8.1.1 The role of Lateral VFA and CT for quantifying VC

The specifically designed preliminary imaging study as detailed in Chapter 3 was aimed to test the hypotheses that lateral VFA imaging – primarily used for the assessment of vertebral deformity – may offer a quick and reproducible method for quantifying AAC which could be implemented into routine clinical practice and performed concurrently with bone density scans in women referred for OP screening. Furthermore, that moderate dose, non-diagnostic, non-contrast CT may also provide a reproducible alternative to higher dose diagnostic CT methods that are routinely used in clinical practice for quantifying coronary VC. Research has shown that most individuals in later life – from the sixth decade onwards – will have evidence of calcium deposition within the major arteries leading to an increased risk of CV events and subsequent significant morbidity and mortality, and moreover VC is frequently observed in patients with OP. This research study demonstrated that lateral VFA imaging may indeed offer a reproducible imaging method to quantify AAC however, for the use of lateral VFA scan images to be expedient in clinical practice, it is important that the process be quick and reliable. The lateral VFA inter-rater agreement data reported in Chapter 3 highlighted the importance of training required to adequately interpret and evaluate the scan images to ensure results are reported accurately and consistently between multiple raters and limit delay and the possibility of the misinterpretation of image artefacts. The preliminary imaging study CT reproducibility data demonstrated that the modified scanning protocol developed specifically for this research study does provide highly reproducible results which confer a safer alternative to the high radiation dose methods currently in use for

evaluating coronary calcification in clinical practice. Additionally, the modified CT scan protocol was suitable for quantifying VC at multiple vascular sites and results demonstrated that scan images can be interpreted and evaluated by an appropriately trained clinical scientist and/or experienced radiographer, and therefore remove the burden of extra scan reporting time imposed on a radiologist. The cross-sectional study results as presented in Chapter 4 further tested the reliability of lateral VFA scan images for quantifying calcification, evaluating its sensitivity and specificity by comparing to the gold-standard of CT. This was an important aspect of the study as there is very limited information published on the accuracy of lateral VFA for the prediction of VC in lower risk populations as the available data are limited to higher risk study populations with renal disease that will have a greater incidence and extent of VC. Results from this study showed that lateral VFA scan images were only able to predict high levels of AAC as detected by CT with moderately-good accuracy and may therefore be limited in any potential clinical application. However, this was not necessarily considered a negative finding. Due to cost, risks associated with radiation exposure and the potential burden on the health service it would be considered prudent in clinical practice to limit the use of imaging techniques used to quantify VC to patients who are at a higher risk for future CV events and who would benefit from further clinical intervention and management.

8.1.2 Relationship between BMD, regulators of bone remodelling and AC/VC

It is now well established that OP and CVD are common age-related conditions and although traditionally, the two conditions were considered unrelated and their coexistence attributed to independent age-related processes, increasing biological and epidemiological evidence suggests that there are age-independent associations between BMD, VC, atherosclerotic burden and CV-related events, morbidity and mortality. The cross-section data from this research was used to investigate the relationship between BMD and AC by evaluating associations between BMD measured at the lumbar spine, femoral neck and total hip with AC measured using lateral VFA scan images and VC at multiple sites using the gold-standard CT. Notwithstanding significant results reported by others, the results of this study failed to find any significant associations between BMD and measures of VC. These results were surprising, particularly in view of the size of the study population data set which included over 400 postmenopausal women. Indeed limitations of imaging methods likely provide the explanation as to why this study found no significant relationship between BMD and AC/VC. As highlighted in Chapter 5 and preceding chapters, quantitative analysis of lateral VFA scan images is significantly affected by patient size and the presence of

image artefacts and furthermore, the quantification of AAC on lateral VFA scan images can be highly subjective and relies on the skill and interpretation of the reader. Another important consideration is that CT imaging methods (like most imaging methods) are currently unable to distinguish between the different types of VC including intimal and medial calcification, so there was no way of determining which type of calcification was being identified, and whether the method used was more sensitive to one type of calcification over another.

Prospective studies have shown AC to be related to bone loss and an increase in fracture risk, and animal studies have shown that many important biochemical factors involved in the regulation of bone and mineral metabolism may also be implicated in the pathogenesis of VC, which provided the rationale to investigate the associations between regulators of bone remodelling with VC and BMD in the present study. Chapter 6 reports results of the association between circulating Wnt inhibitors sclerostin and Dkk1 with AC, VC and BMD which are of particular interest as relationships with Dkk1 and sclerostin may provide further evidence of their role in the pathogenesis of OP and VC, both of which are frequently seen in the ageing female population. Furthermore, there are limited data in the literature regarding the associations between sclerostin and VC in humans and although these studies have demonstrated both a positive and inverse relationship between sclerostin and VC, the majority have been limited to higher risk populations with renal disease and DM that have a greater incidence and severity of VC. Data from this research study demonstrated that serum Dkk1 was not associated with BMD or AC/VC but sclerostin on the other hand was significantly associated with BMD but like Dkk1 was not associated with AC or VC. Only a limited number of subjects were included in this particular study as described in Chapter 6 which may account for the lack of significant results, and as previously reported there are also limitations associated with the imaging methods used to quantify AC/VC. Further large-scale observational and prospective epidemiological studies are required to fully establish the relationships between BMD and VC.

8.1.3 Relationships between PWV, BMD, regulators of bone remodelling and VC.

Pulse wave velocity is a simple, non-invasive method of measuring arterial stiffness and has been shown to be an independent predictor of CV-related events and mortality. It is in part dependent on atherosclerotic burden and/or medial calcification however the relationship between PWV and BMD is less apparent. This study evaluated the relationships between arterial stiffness with BMD, AV/VC and regulators of bone remodelling but unlike results reported elsewhere the results of this study showed no

significant associations between PWV with BMD measured at multiple sites. Furthermore, there was no association between arterial stiffness and Dkk1 or sclerostin – regulators of bone remodelling. As highlighted in Chapter 5 previous studies have reported inconsistent results regarding associations between PWV and BMD with some reporting positive associations while others report no association. The reason for the disparity with reported results is not clear but the use of different imaging methods to measure BMD may account for this. For example, DXA is the most widely used method for measuring BMD however previous studies have used QCT – a superior method of quantifying BMD – which provides a true volumetric measure of bone which cannot be achieved using DXA. This study did not find any association between arterial stiffness measured using PWV with AAC measured using lateral VFA scan images but does initially report a significant association between PWV and VC measured using CT, however this relationship was later found to be confounded by other factors such as age, mean arterial pressure, hypertension and body mass index which have all been significantly associated with arterial stiffness in this and other research areas. This research study also evaluated associations between regulators of bone remodelling – circulating Wnt inhibitors sclerostin and Dkk1 – with arterial stiffness as detailed in Chapter 6 but reported no association although there was a trend for higher sclerostin levels in women with increased arterial stiffness, a finding that has been previously reported by others. The role of circulating sclerostin and Dkk1 in the development or inhibition of VC and arterial stiffness is a relatively understudied area and there are limited data in the literature regarding the associations between circulating sclerostin and Dkk1 levels with arterial stiffness in healthy, ambulatory populations, and whether arterial stiffness and its contributing factors lead to an increase in circulating sclerostin, or increased circulating sclerostin levels result in a reduction of arterial compliance and a subsequent increase in PWV remains to be determined. Ultimately further observational studies on larger study populations would be required to elucidate the relationships between arterial stiffness, BMD and AC/VC.

8.1.4 Bisphosphonates and the effects on AC/VC and aortic stiffness.

In view of the common underlying mechanisms between VC and bone formation there is significant interest in the role of therapies used to treat OP in postmenopausal women and the potential therapeutic effects they may have on VC. It is hypothesised that agents such as BPs that are widely used to treat OP may slow the rate of progression or even inhibit VC. Experimental and animal studies have shown BPs to have a consistent effect on inhibiting VC, however human studies to date have been unable to provide

unequivocal evidence supporting the inhibitory effect of BP. This research project aimed to test the hypothesis that BPs have an inhibitory effect on VC using cross-sectional data and also from data acquired from the randomised controlled treatment study that ran concurrently. This area of research is clinically important as there are currently no therapies available that can reverse VC. Chapter 7 reports on the associations between VC, aortic stiffness and BP use from the cross-sectional study. Unlike previously reported results by others the cross-sectional study results failed to show any association between the use of BP therapy with AC. This was mainly attributed to possible limitations with imaging methods as previously described and the differences in sample size between studies and any possible association between arterial stiffness and BP use was confounded by factors associated with arterial stiffness including age and hypertension as previously discussed. Although not presented here, results of the RCT also failed to show any significant associations between BP use, AC and arterial stiffness although this was likely due to the significantly small sample size. It is important to highlight that the pilot RCT started only after the PhD research project had commenced and was therefore limited by a small sample size that was not sufficiently powered to detect statistically significant change in the outcome measures. Due to the significant limitations the RCT data was not included although it served as a useful adjunct to the results of the cross-sectional study and results from 24-month data will be reported once the RCT study is completed.

Although this research study employed a comprehensive and stringent list of exclusion criterion for the selection of suitable research subjects, as detailed in Section 2.4.1, Chapter 2, it did not specify that subjects would not be eligible for enrolment had they ever used oestradiol and/or testosterone implants or used percutaneous testosterone within the preceding 3-months. However, each study subject completed a bone health questionnaire and was interviewed in order to obtain a comprehensive medical history. It is therefore important to note that none of the subjects enrolled onto the study reported using these hormone preparations.

This research project highlighted the importance of patient education regarding the use and safety of therapies used to treat OP. For example, many volunteers were reluctant to take part in the RCT due to fears surrounding the use of calcium supplementation as a result of the reported association between calcium use and CVD frequently published in the media. Furthermore, there was a great lack of understanding with regard to the clinical application of BPs and their association with incident oesophageal cancer and osteonecrosis of the jaw. Negative reporting in the media and the dissemination of misinformation in various social settings had a great impact on the recruitment for the

RCT resulting in women not wanting to take part for fear of developing cancer and/or developing heart disease as a result of taking BP and/or calcium supplementation. In conclusion, further large-scale RCTs are required to fully evaluate the vascular effects of BPs to establish whether they can provide a concurrent therapy that not only treats OP but may also halt the progression of VC in populations that have an increased clinical risk of CV morbidity and mortality.

Appendix

Abstracts and Publications

Edwards S, Fogelman I, Frost M; The prevalence of abdominal aortic calcification assessed using lateral vertebral fracture assessment DXA scans in postmenopausal women with and without osteoporosis. *Osteoporos Int* (2009) 20 (Suppl 4):S257–S314 S307

Edwards S, Fogelman I, Frost M; The relationship between aortic stiffness and abdominal aortic calcification measured on spine DXA VFA scans in postmenopausal women. *Osteoporos Int* (2012) 23 (Suppl 5): S520 – S551 P6

Frost M, Edwards S; Osteoporosis and vascular calcification. *Osteoporosis Review* (2012) 20:1; 10-12

Hampson G, Edwards S, Conroy S, Blake G M, Fogelman I, Frost M L: The relationship between inhibitors of the Wnt signalling pathway (Dickkopf-1(DKK1) and sclerostin), bone mineral density, vascular calcification and arterial stiffness in postmenopausal women. *Bone* (2013); 56:42-47

Edwards S, Fogelman I, Hampson G, Blake GM, Moore A, Frost ML: Aortic calcification and bone mineral density; A cross-sectional study of healthy postmenopausal women. *Osteoporos Int* (2014)

Ishtiaq S, Edwards S, Sankaralingam A, Evans BAJ, Frost M, Fogelman I, and Hampson G: The effect of nitrogen containing bisphosphonates, zoledronate and alendronate, on the production of pro-angiogenic factors by osteoblastic cells. *Cytokine* 71 (2015) 154–160.

Hampson G, Edwards S, Sankaralingam A, Harrington D J, Voong K, Fogelman I, and Frost ML: Circulating concentrations of vitamin E isomers: Association with bone turnover in post-menopausal women. *Osteoporos Int* (2014) 25 (Suppl 6):S659–S726.

Cecelja M, Edwards S F, Moore A E, Fogelman I, Chowienzyk P, Frost M L. A pilot study to assess effects of alendronic acid on aortic calcification and stiffness in postmenopausal women. (2015) European Society of Hypertension, Poster presentation.

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